Antigenicity and immunogenicity of HIV-1 gp140 with different combinations of glycan mutation and V1/V2 region or V3 crown deletion

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Abbreviations: HIV, Human immunodeficiency virus; Env, envelope glycoptotein; CD4-BS, CD4-binding site; SEC, size exclusion chromatography; bNAb, broadly neutralizing antibody; non-NAb, non-neutralizing antibody; CDR-H3, the third complementarity determining region of antibody heavy chain; ADCC, antibody-dependent cell-mediated cytotoxicity; ELISA, enzyme-linked immune-absorption assay.

Abstract

The carbohydrate moieties on HIV-1 envelope glycoprotein (Env) act as shields to mask conserved neutralizing epitopes, while the hyperimmunogenic variable regions are immunodominant in inducing non-neutralizing antibodies, representing the major challenge for using Env as a vaccine candidate to induce broadly neutralizing antibodies (bNAbs). In this study, we designed a series of HIV-1 gp140 constructs with the removal of N276/N463 glycans, deletion of the V1/V2 region and the V3 crown, alone or in combination. We first demonstrated that all the constructs had a comparable level of expression and were mainly expressed as trimers. Following purification of gp140s from mammalian cells, we measured their binding to bNAbs and non-NAbs in vitro and capability in inducing bNAbs in vivo. Antibody binding assay showed that removal of N276/N463 glycans together with the deletion of V1/V2 region enhanced the binding of gp140s to CD4-binding site-targeting bNAbs VRC01 and 3BNC117, and CD4-induced epitopes-targeting non-NAbs A32, 17b and F425 A1g8, whereas further deletion of V3 crown in the gp140 mutants demonstrated slightly compromised binding capability to these Abs. Immunogenicity study showed that the above mutations did not lead to the induction of a higher Env-specific IgG response via either DNA-DNA or DNA-protein prime-boost strategies in mice, while neutralization assay did not show an apparent difference between wild type and mutated gp140s. Taken together, our results indicate that removal of glycans at N276/N463 and deletion of the V1/V2 region can expose the CD4-binding site and CD4-induced epitopes, but such exposure alone appears incapable of enhancing the induction of bNAbs in mice, informing that additional modification or/and immunization strategies are needed. In addition, the strategies which we established for producing gp140 proteins and for analyzing the antigenicity and immunogenicity of gp140 provide useful means for further vaccine design and assessment.

Key words: HIV-1; gp140; glycan; variable region; antigenicity; immunogenicity

Introduction

Vaccines are believed to be the ideal strategy to prevent infectious diseases, but an effective vaccine against HIV-1 remains elusive. As the key component in viral entry, HIV-1 envelope glycoprotein (Env) represents the primary candidate for vaccine design. However, attempts using Env-based immunogens to induce broadly neutralizing antibodies (bNAbs) against main circulating HIV-1 strains have not been successful in various models (1, 2), whereas such bNAbs have been continuously identified in HIV-1 infected individuals including elite controllers (3). With the advances in understanding Env structure and recognition epitopes of the identified bNAbs, it is believed that Env-based immunogens, once appropriately optimized, may be able to elicit bNAbs in vivo.

Env is first synthesized as a precursor gp160, and then cleaved into noncovalently associated gp120 and gp41 on the viral membrane as heterogenous trimers. Removal of the cleavage site by mutation generates trimeric gp160 while introducing a stop codon to the end of gp41 ectodomain can yield soluble gp140 (4). The trimeric form of Env is believed to be a better vaccine candidate than the corresponding gp120 monomer because non-neutralizing epitopes exposed in the monomer are shielded in the trimer. (5). The surface unit gp120 can be divided into 5 conserved (C1-C5) and 5 variable (V1-V5) regions. In general, conserved regions are believed to be crucial for viral fitness and therefore remain relatively constant across strains. Conserved regions are usually shielded by glycosylation which prevents these sites from recognition by the host immune system. By contrast, variable regions are more dispensable

for viral entry and can vary significantly between different strains without severe impact on viral fitness. Furthermore, variable regions are prone to be immunodominant and the immune responses diverted to these regions are usually non-neutralizing, representing one of the mechanisms that the virus evades the immune system.

Given the importance of glycosylation and variable regions on viral immune evasion, removal of glycans or/and variable regions of HIV-1 Env may represent a feasible approach to induce broadly neutralizing immune responses. We and others previously found that removal of certain specific glycans near CD4-binding site (CD4-BS) on Env not only rendered HIV-1 more sensitive to bNAbs, but also enhanced bNAb induction in animals (6-9). More importantly, a recent study reported that removal of two conserved glycans at N276 and N463 could allow Env-binding to, and activation of, B cells expressing the germline-reverted BCRs of two potent bNAbs VRC01 and NIH45-46 targeting CD4-binding site (10, 11). In addition, genetic removal of the V1/V2 loop was shown to associate with enhanced neutralization of virus by antibodies targeting CD4-binding site and CD4-induced epitopes (CD4i) that overlap with the conserved coreceptor binding site in the bridging sheet (12). In an HIV-1 gp140 vaccine study, a predominant anti-V3 non-neutralizing IgG response was observed, indicating that V3 may play an important role in HIV-1 immune evasion (13).

In this study, we designed, expressed and purified gp140s bearing N276/N463 glycan mutations, V1V2 and V3 deletion, alone or in combination. We compared the antigenicity and immunogenicity of the gp140s using both in vitro and in vivo assays.

Materials and methods

Cells

Cell lines 293T and TZM-bl were obtained from the American Type Culture Collection (ATCC) and NIH AIDS Reagent Program, respectively. Cells were grown in Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) and penicillin-streptomycin (both at 100 U/ml) at 37°C with 5% CO_2. The FreeStyle™ 293-F cell line, purchased from Thermo Fisher Scientific, was grown in FreeStyle™ 293 Expression Medium in a shaking incubator at 37°C with 5% CO_2.

Env constructs

Human codon-optimized CN54 (Clade CRF_07 BC) gp140 in pcDNA3.1(+) was described previously (14, 15) and designated as gp1 in this study. C1 and D1 were obtained by replacing the V3 crown with poly G and a Clade D V3 in gp1, respectively. gp2, C2 and D2 were generated by introducing the N276D/N463D mutations to gp1, C1 and D1, respectively. gp3, C3 and D3 were generated by introducing the V65C/S115C mutations and deleting the V1/V2 loops (124aa-196aa in HXB2) of gp2, C2 and D2, respectively. gp4, C4 and D4 were generated by removing the V3 crown (302aa-311aa in HXB2) of gp3, C3 and D3, respectively. A flow chart of plasmid construction is shown in Figure 1B.

Production and purification of trimeric HIV-1 gp140 proteins

Trimer HIV-1 CN54 gp140 proteins were expressed in 293F cells, with ~360 million cells being transfected with 360 μg DNA, and purified by lentil lectin affinity chromatography followed by size exclusion chromatography (SEC), as described previously with modifications (16, 17). Briefly, 293F cells at a density of 120,000 ~160,000 viable cells/ml were transfected with gp140-expressing plasmids (1 μg /one million of cells) using

polyethyleneimine (PEI) (4 μg/1μg plasmids). Five days after transfection, cell culture supernatants were harvested and loaded onto a pre-equilibrated lentil lectin column (GE Healthcare). After washing with 5 column volumes of binding buffer (0.5 M NaCl, 20 mM Tris, 1mM $MnCl_2$, 1mM $CaCl_2$, pH 7.4), gp140 proteins were eluted with 10 column volumes of elution buffer (0.5 M NaCl, 20 mM Tris, 0.4 M α-D-methylglucoside). The eluted proteins were concentrated using 100-kD columns (Millipore) and trimeric gp140 proteins were further purified using SEC with a Superose 6 prep grade HR 16/50 column (GE Healthcare). Protein concentration was determined by BCA assay according to the manufacturer's instructions (Thermo Scientific).

SDS-PAGE, blue native-PAGE (BN-PAGE), Western blot and silver staining

SDS-PAGE was performed as described previously (18). Briefly, cell culture supernatants or purified protein samples were prepared in loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol) and boiled for 10 min. Gel electrophoresis was performed for 0.5 h at 60 V and 1.5 h at 120 V using Tris/Glycine/SDS buffer (25 mM Tris, 0.25 M Glycine, 0.1% SDS). BN-PAGE was performed as described previously (18). Culture supernatants or purified protein samples were prepared in 2 × Protein Native PAGE Loading Buffer (TaKaRa), and loaded onto a 4 to 12% Bis-Tris NuPAGE gel (Invitrogen). Typically, gel electrophoresis was performed for 2 h at 200 V using electrophoresis buffer (50 mM MOPS, 50 mM Tris, pH 7.7). Western blot analysis was performed as described elsewhere (18). Following transfer, the polyvinylidene difluoride (PVDF) membrane was blocked with 5% non-fat milk, and then probed using anti-HIV Immune Globulin (NIH AIDS Reagent Program), followed by HRP-conjugated rabbit anti-human IgG (Boster biological technology). Silver staining was done with Sangon silver dye kit according the manufacturer's instructions (Sangon Biotech).

Bio-layer interferometry analysis of mAb-gp140 binding

Monoclonal antibodies (mAbs) against different epitopes of HIV-1 Env were obtained from the NIH AIDS Reagent Program, including: VRC01, 3BNC117 (against CD4 binding site); 17b, F425 A1g8, A32 (against CD4i epitope); 3869 (against V3 region). Bio-layer interferometry analysis of mAb-Env binding was performed on the Octet KQe instrument (ForteBio, Inc.), as described previously with modifications (10). Briefly, mAbs (5 μg/ml in PBS) were first immobilized onto anti–human IgG Fc capture (AHC) biosensors (ForteBio) for 600s, and then the baseline interference was read for 600s in kinetics buffer (KB: 1× PBS, 0.05% BSA, 0.02%, Tween 20, pH 7.4). Subsequently, the sensors were immersed into trimeric gp140 diluted in KB for 600s (association phase), followed by immersion in KB for the indicated times (dissociation phase). All kinetic interactions were measured with new sensors at 30°C and 1,000 rpm in 96-well plates. Curve fitting was done with a 1:1 binding model using the software ForteBio. Mean k_{on} and k_{off}, and apparent K_d values were calculated from all binding curves with an R^2 value ≥ 0.95 (19).

Ethical statement and mouse immunization.

All animal experiments were approved by the Institutional Ethical Review Board of the Wuhan Institute of Virology, Chinese Academy of Sciences, and handled in accordance with the guidelines of the Hubei Laboratory Animal Science Association. Six-to-eight weeks old, specific-pathogen-free (SPF) female BALB/c mice were obtained from the Beijing Vital River Laboratory of Animal Technology and hosted in an SPF environment with food and water supplied. DNA-DNA and DNA-protein prime-boost strategies were adopted in this study. For DNA immunizations, mice were injected with 30 μg plasmids each intramuscularly

with electroporation. For protein immunizations, mice were injected with Freund's complete adjuvant formulated CN54 gp140 protein (7 μg/animal) intraperitoneally. All immunizations were given 3 times in 2-week intervals. Serum samples were collected 7 d after the final immunization and used for ELISA and neutralization assay.

Enzyme-linked immune-absorption assay (ELISA).

Microlon 96-well plates were coated overnight with trimeric CN54 gp140 at 5 μg/ml in cold PBS. After washing and blocking steps, serially diluted mouse sera were added at 50 μl/well and incubated for 1 h at 37 °C. Unbound antibodies were washed away with PBST (PBS/0.05% Tween-20). Horseradish peroxidase (HRP) labeled goat-anti-mouse immunoglobulin G (IgG) was added at a 1:8000 dilution in PBST and incubated for 1 h at 37 °C. After 5 washes with PBST, colorimetric reaction was performed using TMB for 5 min in the dark and stopped using 2 M H_2SO_4. Absorption was measured at 450 nm (test wavelength) and 570 nm (reference wavelength). End point titers were calculated with a cut-off value defined as the mean of OD value from naïve murine sera plus 2 SD.

Virus production and titration

Pseudotyped virus was produced by co-transfecting 293T cells with pCN54 gp160 and the HIV-1 backbone plasmid, pSG3ΔEnv. Forty-eight hours post transfection, virus-containing culture medium was harvested and FBS concentration was adjusted to 20%. After filtration through a 0.45 μm micron filter, the virus stocks were aliquoted and stored at -80°C. Titration of the virus stocks was done using the TZM-b1 system, and 50% tissue culture infection doses ($TCID_{50}$) was calculated.

TZM-bl neutralization assay

TZM-bl neutralization assay was performed as described previously (20, 21). Briefly, 1 day prior to infection, 5000 TZM-bl cells per well were seeded in a 96-well plate in DMEM containing 10% FBS, penicillin and streptomycin (both at 100 U/ml), and incubated at 37˚C with 5% CO_2 for 48 h. Fifty $TCID_{50}$ virus was first incubated with serially diluted heat inactivated sera for 1 h at 37˚C, and then the virus-serum mixture together with 40 mg/ml DEAE was added into the pre-seeded TZM-b1 cells. After 48 h, the medium was removed, and the cells were washed, lysed and luciferase activity was measured using a Luciferase Assay kit, according the manufacturer's instructions (Promega). All conditions were done in duplicate. Background luciferase activity was subtracted with luciferase activity from uninfected cells. The serum neutralization activity was expressed as the percentage of virus inhibition, with the neutralization activity of the pcDNA3.1(+) group being set to 0% (5).

Statistical analysis

All statistical analyses were performed with GraphPad Prism 7. For comparisons between two groups, student's t test was adopted, while for comparisons among three and more groups, One-way ANOVA plus SNK *post hoc* was used. A p value less than 0.05 was considered statistically significant.

Results

Production of stable homogeneous gp140 trimers.

Based on the trimeric CRF_07 BC CN54 gp140-expressing plasmid (gp1) as previously reported (14, 15, 22-24) (Figure 1A), we designed and constructed a series of plasmids with N276D/N463D glycan mutations, V1V2 region and V3 crown deletion, alone or in

combination, named gp2, gp3, gp4, C1, C2, C3, C4, D1, D2, D3 and D4, respectively (Figures 1A and 1B). The flow chart for construction of the above plasmids is shown in Figure 1B. The expression of the constructs was analyzed by transient expression in 293T cells, followed by SDS-PAGE/Western blot and BN-PAGE/Western blot of 20 µl culture supernatants each, respectively. As shown in Figure 1C, all constructed plasmids were expressed efficiently in 293T cells. Furthermore, BN-PAGE analysis showed that, despite different modifications being made, all the gp140s were mainly expressed as trimers (Figure 1D).

The gp140 proteins were produced in 293F cells and then purified by lentil lectin affinity chromatography followed by size exclusion chromatography (SEC) to obtain the trimeric forms. As shown in Figure 1E, gp140 trimers were well separated from aggregates, dimers and monomers. BN-PAGE (Figure 1F), SDS-PAGE (Figure 1G), and silver staining (Figure 1H) of the BN-PAGE gels showed that the purified gp140 proteins were mostly trimers. Data of gp1 are shown in the figure as an example since similar results were observed for all the purified gp140 mutants. The yields of all the constructs were comparable (Table S1).

Antigenic analysis of gp140 trimers by bio-layer interferometry.

We next investigated the binding of gp140s with a range of bNAbs and non-NAbs using bio-layer interferometry. In this assay, we divided the trimers into 3 groups. The responses of different gp140s to different Abs are shown in Table 1. We found that VRC01 and 3BNC117 (Abs targeting the CD4 binding site) had the strongest binding to gp3, C3 and D3 (mutants with V1/V2 deletion). However, the binding of the same mAbs to gp4, C4 and D4 was relatively weak, but still stronger than that to gp1, C1 and D1 (Figure 2A and Table 1).

Similarly, the binding of CD4-induced epitopes (CD4i) targeting mAbs (A32, 17b and F425 A1g8) to gp4, C4 and D4 was inferior to that to gp3, C3, and D3, but stronger than that to gp1, C1 and D1 (Figure 2B and Table 1). Not surprisingly, mAb 3869 targets V3 crown had no apparent binding to mutants with V3 crown deletion (gp4, C4 and D4) (Figure 2C and Table 1). These data herein indicate that V1/V2 deletion together with N276/N463 glycan removal exposes CD4-binding site and CD4i epitopes, while further deletion of V3 crown may impair the stability of gp140 protein.

Immunogenicity of gp140 trimers in mice.

To evaluate whether removal of glycans or/and variable regions would enhance the induction of bNAbs, the immunogenicity of the modified gp140s were tested on a BALB/c mouse model. Two immunization strategies, DNA-DNA and DNA-protein prime-boost strategies, were adopted in this study. For DNA-DNA prime-boost, mice were injected with 3 injections of DNA in 2-week intervals, whereas for DNA-protein prime-boost, mice were primed with DNA and boosted with two additional injections of proteins in 2-week intervals. Seven days after the final immunization, sera were collected to determine gp140-specific antibody titer (ELISA) and viral NAbs titer (neutralization assay). Our results showed that DNA-DNA prime-boost only induced moderate antigen-specific IgG response (Figure 3A), while DNA-protein prime-boost significantly enhanced antibody production. However, compared to wild type gp140, all the mutants did not induce higher antigen-specific IgG production in mice using both immunization strategies.

We then tested the neutralization activities of sera from DNA-protein immunized mice against homologous virus CN54. The neutralization activities of all the tested groups were at

a similar level, indicating that such modifications could enhance the binding of gp140 to bNAbs but could not efficiently induce the induction of bNAbs in mice (Figure 3B). Neutralization assay against heterologous viruses BaL and NL4-3 was also tested and no apparent difference was observed (data not shown).

Taken together, the above results indicate that glycan removal at N276/N463 together with V1/V2 deletion on gp140 enhanced the exposure of CD4-binding and CD4i epitopes but such modifications alone seem unable to efficiently increase bNAb induction in mice.

Discussion

Although bNAbs are believed to have the potential to provide complete protection against HIV-1 infection, there are considerable challenges in inducing such antibodies by vaccination. Given that the conserved epitopes which bNAbs bind to are masked in native Env (25), a number of studies have been focusing on HIV-1 Env modification to induce bNAbs in vivo (4, 12, 26). Previous studies have showed that removal of two conserved glycosylation sites N276 or N463, located in loop D and variable region 5, may facilitate the induction of CD4BS bNAbs (10). In addition, it was reported that viruses with genetic removal of the V1/V2 loop were associated with enhanced neutralization by antibodies against CD4 binding site and CD4i epitopes (12, 27). Most Abs raised during the early phase of HIV-1 infection are against neutralization-irrelevant epitopes on gp41 and the V3 region of gp120 (28, 29). It remains unknown whether, and to what degree, “off-target” epitopes for non-NAbs would interfere with the more desirable bNAb responses. Furthermore, whether a reduction in inducing “off-target” Ab responses, or/and whether an increased exposure of conserved epitopes, could have a beneficial effect on the development of bNAbs, has yet to

be determined.

In the current study, we introduced a series of modifications to the CN54 gp140 and investigated the impact on gp140 antigenicity and immunogenicity. The antigenicity study indicated that removal of glycans at N276 and N463 only slightly enhanced the exposure of CD4 binding and CD4i epitopes, while a significantly greater exposure was detected when further deletion of the V1V2 loop was introduced. However, further deletion of the V3 crown appeared to impair the exposure of gp140 to CD4 binding and CD4i epitopes. As reported by others, the deletion of the V3 loop induces conformational changes in the CD4 binding region of gp120 due to the relationship between the V3 loop and the fourth conserved (C4) region of the gp120 glycoprotein (30, 31). V3 extends outward from the crown to a conserved and exposed glycan at N332, which is a target of highly potent bnAbs including PGT121-122, PGT126 and PGT128. The binding of PGT126 to gp4, C4 or D4 was decreased moderately compared with that to gp3, C3 or D3 (Figure S1), suggesting that V3 deletion may affect the conformation of the gp140 trimers. The results are consistent with those in Figure 2, showing that the effect of the V3 deletion lead to the moderate decrease of binding to VRC01 and A32. These findings together reveal that, although certain modifications can increase the exposure of CD4BS on gp140 targeting by bNAb, such modified immunogens alone are unable to induce high level of neutralizing Abs in mice. Additional modification or/and immunization strategies may be warranted.

Previous studies have revealed that gp140 trimer with N276D and N463D mutations confers binding to human germline-reverted NIH45-46 and VRC01, and may facilitate the induction of CD4BS bNAbs. In addition, removal of the V1, V2, V3 regions reduces the ability of

several anti-CD4 nNAbs to block rENV activation of glVRC01 class B cells, and it also was reported that viruses with genetic removal of the V1/V2 and V3 loop were associated with enhanced neutralization by antibodies against CD4 binding site and CD4i epitopes (10, 27, 32). There are a number of reasons as to why a gp140 immunogen with enhanced neutralizing epitopes and reduced non-neutralizing epitopes was unable to enhance NAb responses in mice. First, in vitro B-cell activation studies suggest that, because of their higher affinity for Env, germline non-NAb precursors have a selection advantage in the germinal center over the germline bNAb precursors. In the context of germinal center, the selection of higher-affinity B cell clones after Env immunization can hinder the activation and affinity maturation of low-affinity germline bNAbs (32-34). It is probable that, although more germline bNAb precursors could be activated by the modified gp140 with enhanced neutralizing epitope exposure, their affinities were still too weak to compete with the remaining non-Nab precursors. Second, the germline precursors of bNAbs require extensive somatic hypermutations to become high affinity bNAbs, which is a long and complex process and is unlikely to be achieved in animal models. In human, less than 5% to 10% of people who are naturally infected with HIV-1 can develop bNAbs, but not until 3 to 5 years after their infection. In our animal experiments, the immune process lasted only approximately two months, likely too short a time for the maturation of the germline precursors of bNAbs. Third, a growing number of studies have pointed out that mice may not be the appropriate model for HIV-1 vaccine study because of their lack of enough B cells, which may result in weak bNAb responses. Furthermore, the third complementarity determining region of antibody heavy chain (CDR-H3) forms the center of the classical antigen-binding site, which plays a dominant role in determining the specificity and affinity of an antibody. However, while the CDR-H3 length has potent influence on the range of antigen-biding structure,

mouse CDR-H3s are significantly shorter than human ones (35-39). Therefore, the constructs made in this study might still merit trying in other animals and possibly humans to examine whether these mutations are advantageous. In addition, our study reveals that removal of V1/V2 loop can enhance the exposure of CD4i epitopes which have been shown to be preferentially targeted by ADCC-mediated antibodies (40-43). Although it is beyond the scope of our current study, it would be interesting to test whether HIV-1 gp140 with V1/V2 deletion would induce higher ADCC responses in different animal models.

In summary, our data demonstrate that removal of glycan at N276/N463 together with deletion of the V1/V2 region facilitated the exposure of the CD4-binding site and CD4-induced epitopes of gp140. Although such exposure was incapable of enhancing NAb induction in mice, the strategies which we established for producing gp140 proteins and for analyzing the antigenicity and immunogenicity of gp140 provide useful means for further vaccine design and assessment.

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Competing financial interests

362 The authors have declared no competing financial interests.

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Table 1. Responses of different gp140s to different Abs.

	gp1	gp2	gp3	gp4	C1	C2	C3	C4	D1	D2	D3	D4
VRC01	0.2405	0.4729	**0.9728**	0.5061	0.5250	0.3400	**0.9190**	0.6746	0.2180	0.1509	**0.9428**	0.7261
3BNC117	0.2543	0.3972	**0.8134**	0.4252	0.6685	0.2397	**0.8675**	0.6957	0.5846	0.2528	**0.9606**	0.7618
A32	N	N	**0.6193**	0.2372	0.1449	N	**0.6739**	0.3680	N	0.1418	**0.7716**	0.6733
F425 A1g8	0.4686	0.1128	**1.1493**	0.8699	0.8290	0.2307	**1.1366**	0.9251	N	0.1435	**1.1905**	1.0120
17b	0.2542	0.1081	**0.9004**	0.5159	N	0.2533	**0.8195**	0.6408	N	N	**0.5578**	0.6310
3869	0.6573	1.1245	1.1977	N	1.1663	0.4540	1.4191	N	0.7155	0.2721	0.9271	N

Binding was measured by bio-layer interferometry. A bigger value indicates a stronger binding between the protein and the antibody. Value in each group was compared with the value of gp1, and the biggest was highlighted in bold. The unit of the measurement is "nm". N, no binding detected.

Figure Legends

Figure 1. Construction of HIV-1 gp140 plasmids and production of gp140 trimeric proteins. (A) Based on HXB2 gp120, the constant regions C1, C2, C3, C4 and C5, and the variable regions V1, V2, V3, V4 and V5 are shown. The changing sites are indicated. (B) The flow chart for construction of the HIV-1 gp140 plasmids**.** (C) SDS-PAGE analyses of the trimers to ensure correct expression. (D) BN-PAGE analyses of the trimers expressed in 293T cells. (E) Biochemical characterization of the gp140 trimers. The gp140 constructs were transfected into 293F cells for expression in FreeStyle™ 293 Expression Medium. Data of gp1 are shown as the example. After purified by lentil lectin affinity chromatography, the eluted samples were concentrated and further purified by SEC on a Superose 6 prep grade HR 16/50 column. (F) Native-PAGE analyses of the trimers eluted from SEC column. The three lanes represent elution samples at different time points under the same peak. (G) SDS-PAGE analyses of the trimers eluted from SEC column. The three lanes represent elution samples at different time points under the same peak. (H) Silver stain analyses of the trimers eluted from SEC column. One representative experiment out of three is shown.

Figure 2. Antigenic comparison of gp140 trimers by bio-layer interferometry. The binding was assessed by capture with the following panels of antibodies, including CD4 binding site directed Abs (VRC01), V3 directed Ab (3869), and CD4-induced epitope directed Abs (A32), respectively. Four concentrations from 6.25 nM to 50 nM were tested in 2-fold serial dilutions, with the concentration of each trimer at 50 nM being chosen to compare the binding response with the corresponding mAb. One representative experiment out of three is shown.

Figure 3. Induction of antigen specific serum IgG in immunized mice**.** (A) Sera of mice, vaccinated with plasmids alone, obtained ten days after the final immunization, were tested by ELISAs against CN54 gp140 trimers. (B) Sera of mice, vaccinated with plasmids and proteins, obtained ten days after the final immunization, were tested by ELISAs against CN54 gp140 trimers. (C) Obtained sera were tested against the homologous CN54 pseudoviruses in TZM.bl neutralization assays. Sample neutralizing activity was expressed as the percentage of virus inhibition, with the positive control (cells incubated with virus only) being set as 0%. Data shown are mean ± SD (n=5 mice/group) of three independent experiments. A p value less than 0.05 was considered statistically significant **P<0.01.

Figure 1

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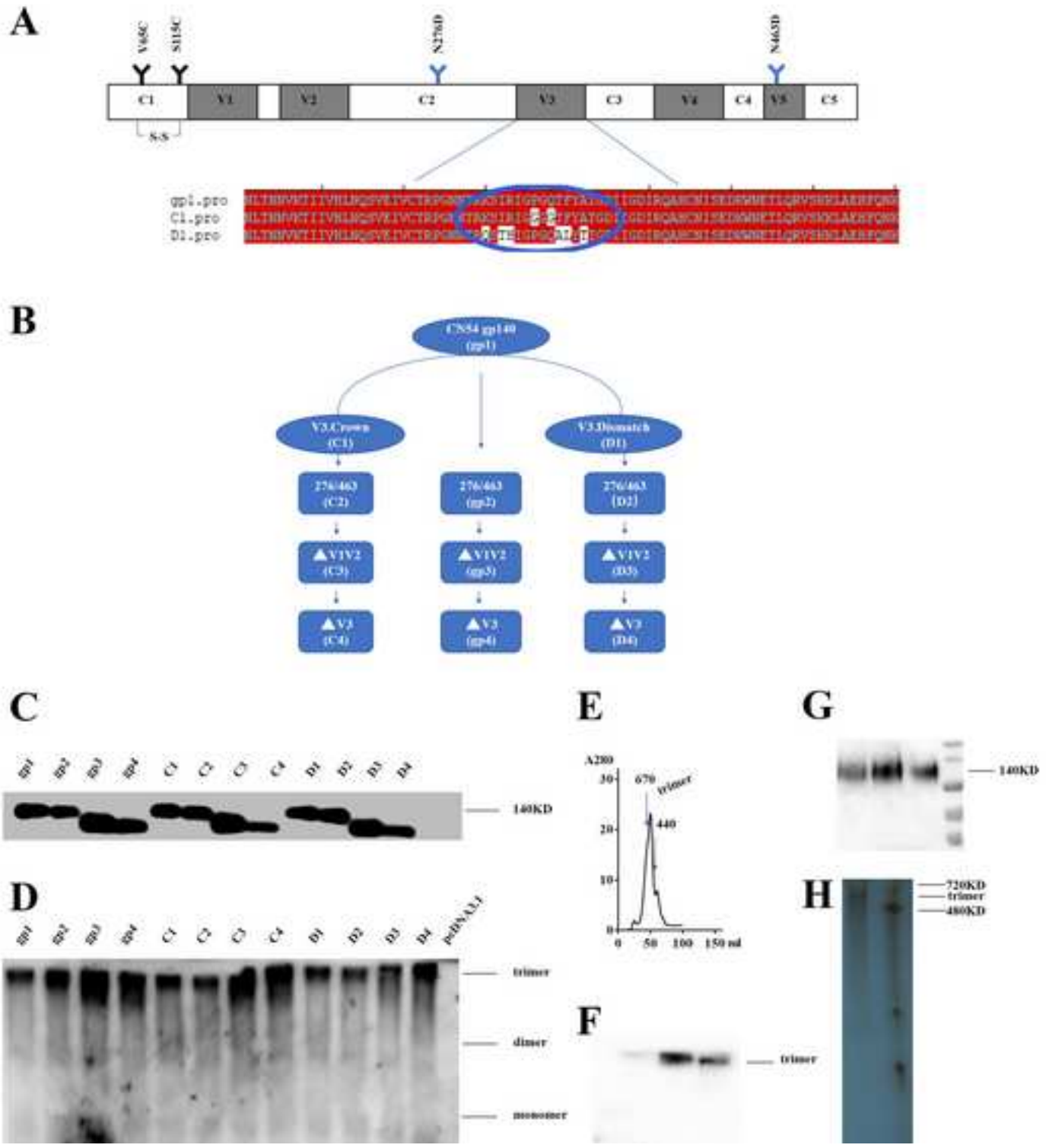

Figure 2

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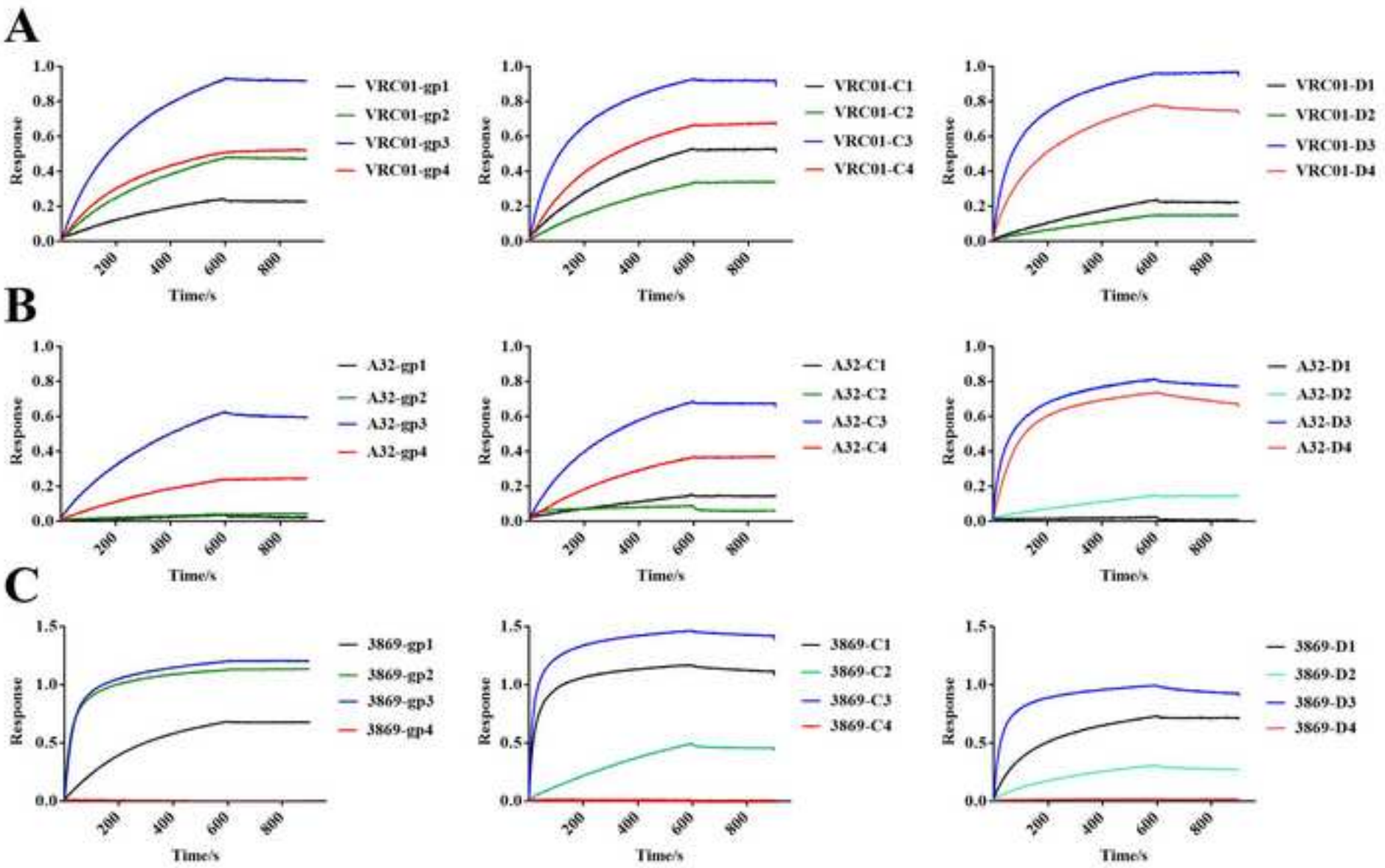

Figure 3

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