HIV-1 Virus Like Particles Produced by Stably Transfected

*Drosophila S2 cells - a Desirable Vaccine Component

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Abstract

The development of a successful vaccine against HIV-1 likely requires immunogens that elicit both broadly neutralizing antibodies against envelope spikes and T cell responses that recognize multiple viral proteins. HIV-1 virus like particles (VLP), because they display authentic envelope spikes on the particle surface, may be developed into such immunogens. However, in one way or the other current systems for HIV-1 VLP production have many limitations. To overcome these, in the present study we developed a novel strategy to produce HIV-1 VLP using stably transfected *Drosophila* S2 cells. We co-transfected S2 cells with plasmids encoding HIV-1 envelope, gag and rev proteins and a selection marker. After stably transfected S2 clones were established, HIV-1 VLP and their immunogenicity in mice were carefully evaluated. Here, we report that HIV-1 envelope proteins are properly cleaved, glycosylated and incorporated into VLP with gag. The amount of VLP released into culture supernatants is comparable to those produced by insect cells infected with recombinant baculoviruses. Moreover, cryo-EM tomography revealed average 17 spikes per purified VLP and antigenic epitopes on the spikes were recognized by broadly neutralizing antibodies 2G12, b12, VRC01 and 4E10, but not by PG16. Finally, mice primed with DNA and boosted with VLP in the presence of CpG exhibited anti-envelope antibody responses including ELISA-binding, neutralizing, ADCC and ADCVI as well as envelope and gag-specific CD8 T cell responses. Thus, we conclude that HIV-1 VLP produced by the S2 expression system has many desirable features to be developed into a vaccine component against HIV-1.
Introduction

Developing a safe and effective vaccine to control HIV-1 pandemic is a major global health priority. The encouraging results from a recent phase III study (RV144) of a combination vaccine regimen conducted in Thailand have created optimism that a preventive vaccine can be developed, although the efficacy of that regimen was judged to be marginal, short-lived and not sufficient to be useful at a population level (40). Thus, optimal vaccine may require a component that elicits broadly neutralizing antibodies that are capable of binding to the envelope spikes on the virion surface as well as memory T cells that recognize multiple T cell epitopes on viral proteins (31).

HIV-1 virus like particles (VLP), because they display authentic envelope spikes on the particle surface, may be developed into such a vaccine component to elicit both neutralizing antibody and memory T cell responses (11, 57, 58). Indeed, immunization of HIV-1 VLP has been shown to generate promising immune responses in animals. For example, Hammonds et al. demonstrated that in a guinea pig model the breadth of neutralizing antibody response elicited with HIV-1 VLP produced by stably transfected 293T cells was enhanced as compared with subunit protein of the same HIV-1 isolate (16). Buonaguro et al. showed that systemic and mucosal cross-subtype neutralizing antibody responses were elicited in mice with HIV-1 VLP produced by insect cells infected with recombinant baculoviruses (RB) (5). McBurney et al. showed that HIV-1 VLP produced by transfected COS cells elicited broader cell-mediated peripheral and mucosal immune responses than polyvalent and monovalent envelope vaccines.
However, in macaque challenge models definitive proof of protection has not been clearly demonstrated. Immunization with SIV/HIV VLP elicited anamnestic response to HIV-1 gp120, which correlated with accelerated clearance of SHIV (34); immunization with single cycle SIV elicited broad SIV-specific T cell responses and significantly reduced viral loads after intravenous SIV challenge (22); repeated vaccination with VSV-G-pseudotyped SIV VLP significantly reduced peak viremia after mucosal SIV challenge, but persistent suppression of viral load was not achieved (25); vaccination with chemically inactivated SIV particles elicited both SIV envelope-specific binding and neutralizing antibody responses and significantly reduced viral loads after intravenous homologous SIV challenge, but failed to resist subsequent heterologous SIV challenge (26); whereas immune responses elicited by VLP alone or by heterologous poxvirus-VLP prime-boost did not protect macaques from SHIV or SIV challenge (33, 50).

Although HIV-1 VLP as immunogens has shown great promising, in one way or the other the production of HIV-1 VLP by current systems have many limitations. For example, yeast (42) or mammalian 293T (16), COS (30) and Vero (36) cells transiently co-transfected with DNA plasmids encoding HIV-1 envelope and gag proteins can produce enough HIV-1 VLP for small animal studies, but not enough for large animals and humans. Because of this, attempts have been made to establish stable mammalian cell transfectants for HIV-1 VLP production, in which genes encoding HIV-1 gag and envelope were driven by a tetracycline-inducible promoter (16, 17). Although this approach yields more HIV-1 VLP, the
amount is still too low to be practical for large animals and humans. Using insect cells infected with RB yields much higher amount of HIV-1 VLP (37, 41). The HIV-1 VLP produced by infected insect cells elicits both humoral and cellular immune responses (5). However, there are three major drawbacks in using insect cells infected with RB for HIV-1 VLP production. First, culture supernatants contain both HIV-1 VLP and RB. Routine purification procedures such as gradient centrifugation or ultrafiltration can not strictly discriminate HIV-1 VLP from RB. As a result, significant amount of live baculovirus is present in the HIV-1 VLP-containing fractions (17, 41). Contamination of HIV-1 VLP preparations with live baculovirus is important with regard to immunogenicity and regulatory issues. Because of this, as an alternative to the baculovirus system, piggyback transposition that expressed HIV-1 gag protein was developed to create transgenic insect cell lines for continuous HIV-1 gag VLP production (27). Second, HIV-1 gp160 precursor envelope proteins are not cleaved in insect cells infected with RB (17), although the effect of uncleaved gp160 on immunogenicity has yet to be investigated. Third, since insect cells infected with RB are short-lived, new infection is required each time when new batch of HIV-1 VLP is going to be produced. Because of this, the amount and the quality of HIV-1 VLP could vary among different batches. Therefore, to overcome the limitations associated with current HIV-1 VLP production systems, in the present study we developed a novel strategy to produce HIV-1 VLP using stably transfected Drosophila melanogaster Schneider 2 (S2) cells. S2 cells have been widely used to produce ectropic proteins,
including HIV-1 envelope proteins (8, 21). HIV-1 envelope precursor gp160 has been shown to be properly cleaved in S2 cells during its biogenesis (4, 21). We hypothesized that S2 cells, due to their high efficiency in expressing ectropic proteins and proper cleavage of HIV-1 envelope precursor, can be used to produce HIV-1 VLP. Furthermore, because the system uses only plasmids, HIV-1 VLP produced by this system will have no recombinant virus contamination. Finally, stably transfected S2 cells can grow to high viable cell density, resulting in high amount of HIV-1 VLP production in culture supernatants. Because of these, stably transfected S2 cells may overcome many limitations and drawbacks in HIV-1 VLP production by the current systems.

To test this hypothesis, we co-transfected S2 cells with plasmids encoding an envelope glycoprotein (consensus B or C), a rev-independent gag (Pr55) protein and a rev protein along with a pCoBlast selection marker. For the comparison, we also co-transfected S2 cells with plasmids encoding a rev-independent gag and a pCoBlast marker or with plasmids encoding gp120 (consensus B or C), a rev protein and a pCoBlast marker. After the stable cell transfectants were generated, limiting dilution assay was performed to establish stably transfected S2 clones. After stably transfected clones were established, expression, process and glycosylation of envelope proteins, production, morphology and antigenicity of HIV-1 VLP, and the immunogenicity of HIV-1 VLP in mice were carefully evaluated.
Materials and Methods

Cell lines

_Drosophila_ S2 cells were provided by Dr. Vincent Deubel at the Institut Pasteur of Shanghai and cultured in complete Express FIVE® SFM medium [i.e. Express FIVE® SFM medium supplemented with 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin and 100 mg/L L-glutamine] at 28°C without CO₂. Every 3 or 4 days the cells were split at the density of 1 × 10⁶ cells per ml.

The human embryonic kidney cell line 293T was purchased from Invitrogen Life Technologies and maintained in complete DMEM medium [i.e. high glucose DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, penicillin (100 U/ml), streptomycin (100 μg/ml)] plus G418 (500 μg/ml) (Invitrogen Life Technologies). TZM-bl cells were obtained from the NIH AIDS Research and Reference Reagent Program (ARRRP, Germantown, MD) (9, 38). Human CD4⁺ T cell line CEMss-CCR5 was generated as described before (54). These cells were maintained in complete DMEM.

Chronically HIV-1-infected CEMss-CCR5 cells were prepared by incubating CEMss-CCR5 cells with a HIV-1 strain AD8 at one multiplicity of infection (MOI) overnight. After the infection, cells were maintained in complete DMEM medium. Periodically, HIV-1 replication and cell surface expression of HIV-1 envelope protein were measured (see below).

Antibodies and pooled HIV-1 patient sera

Human anti-gp120 or gp41 antibodies b12, 2G12, PG16 and 4E10 and mouse anti-gag p24 antibody (183-H12-5C) were obtained from ARRRP. Pooled
HIV-1 patient plasmas were provided by Dr. Ping Zhong at the Shanghai Municipal Center for Disease Control and Prevention, Shanghai, China. Mouse anti-gp120 antibody (cat. #4301) and sheep anti-gp120 C5 capture antibody (cat. #D7324) were purchased from Advanced Bioscience Laboratory INC (Silver Spring, MD) and Aalto BioReagents (Dublin, Ireland), respectively. Human anti-gp120 antibody VRC01 and human anti-H5 HA antibody 100F4 were produced and purified in our laboratory by stably transfected S2 clones as described before (53). APC-conjugated anti-CD8, PerCP-conjugated anti-CD4, FICT-conjugated anti-IFN\(\gamma\), PE-Cy7-conjugated anti-TNF\(\alpha\), purified anti-CD49d and anti-CD28 antibodies were purchased from BD Bioscience (Mountain View, CA).

**Gene constructs**

cDNA sequence encoding HIV-1 rev-independent gag p55 (M1-10) (44) was generated by recursive PCR (39) and cloned into a TA vector (Invitrogen) and sequenced as described before (59). The correct insert was inserted into EcoRI and NotI sites of a constitutive S2 expression vector pAc5.1/V5-His (Invitrogen). The resulting construct was designated as pAC-Gag(Pr55) (Fig. 1A).

cDNA encoding consensus B and C HIV-1 gp160 envelope proteins without a signal peptide were PCR-amplified using HIV-1 consensus B and C envelope genes (23, 24) (generously provided by Dr. B. H. Hahn at the University of Alabama) as templates and inserted into the EcoRI and XhoI sites of an inducible S2 expression vector pMT/BiP/V5-His (Invitrogen). The resulting constructs were designated as pMT/BiP-gp160 (consensus B and C), respectively (Fig. 1A).
cDNA sequence encoding HIV-1 rev was amplified using a pZeoSV/rev as a template and inserted into the EcoRI and NotI sites of an inducible expression vector pMT/V5-His (Invitrogen). The resulting construct was designated as pMT-Rev (Fig. 1A).

cDNA encoding consensus B and C HIV-1 gp120 envelope proteins without a signal peptide were inserted into the BamHI and XhoI sites of an inducible S2 expression vector pMT/BiP/V5-His. The resulting constructs were designated as pMT/BiP-gp120/V5-His (consensus B and C), respectively.

For the mammalian expression, cDNA encoding consensus B and C HIV-1 gp120 envelope proteins with a signal peptide or HIV-1 gag (Pr55) were inserted into mammalian expression vector pCMV/R (3). The resulting plasmids were designated as CMV/R-gp120 (consensus B and C) and CMV/R-Gag (Pr55), respectively. Plasmid was produced at the concentration of 1 mg/ml in sterile water and found to be predominantly supercoiled by gel electrophoresis with endotoxin levels below 1.5 EU/mg DNA.

**Generation of stable Drosophila S2 cell clones**

To generate stable S2 transfectants to produce HIV-1 VLP, S2 cells were co-transfected with 9.5 μg pAc-Gag(Pr55), 4.75 μg pMT/Bip-gp160 (consensus B or C), 4.75 μg pMT-Rev along with 1 μg selection vector pCoBlast which contains blasticidin resistance gene (Invitrogen) using a calcium phosphate precipitation method (47). After overnight incubation, cells were washed once with PBS and cultured in complete Express FIVE® SFM medium. Seventy two h later blasticidin (Invitrogen) at the final concentration 25 μg/ml was added and stable S2
transfectants were generated in 2 to 3 weeks.

To generate stable S2 clones, limiting dilution assay was performed as described before (54). Briefly, $1.5 \times 10^2$ S2 transfectants were mixed with $1 \times 10^6$ parental S2 cells (feeder layer cells) in 10ml *Drosophila* Schneider 2 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin and 100 mg/L L-glutamine. 100 μl cell mixture was added into each well in 96-well plates and incubated at 28°C for 2 days. Blasticidin at the final concentration 25 μg/ml was then added into the culture medium. After 3 weeks, single clones were isolated for further expansion. The stably transfected S2 clones were induced by 5 μM of CdCl2 and the expression of HIV-1 envelope and gag proteins in culture supernatants were analyzed by western blot analysis (see below). The S2 clones that produced the highest HIV-1 VLP (consensus B and C) were selected for HIV-1 VLP production (see below).

To generate stably transfected S2 cells expressing soluble gp120, S2 cells were co-transfected with 9.5 μg pMT/BiP-gp120/V5-His (consensus B or C), 9.5 μg pMT-Rev along with 1 μg selection vector pCoBlast and selected with blasticidin as described above. After stable transfectants were established, limiting dilution assay was performed as described above. The S2 clones that produced the highest gp120 were selected for gp120 production (see below).

**Production of HIV-1 VLP and soluble gp120 by S2 clones**

Wave bioreactor 20/50EHT systems with a WAVEPOD Process Control Unit (GE Healthcare) were used to grow S2 clones in fed batch culture for HIV-1 VLP and soluble gp120 production as described before (53). Briefly, $6 \times 10^8$ cells of
the S2 clones in 300 ml of complete Express FIVE® SFM medium were inoculated into a 2-L cellbag and cultured at 28°C without CO₂. At 5, 7 and 8 days after the initial culture in the Wave bioreactor 300, 200 and 200 ml of fresh complete Express FIVE® SFM medium were added, respectively. At 8 days after the initial culture CdCl₂ at the final concentration of 5 μM was added into culture medium to induce the expression of HIV-1 envelope proteins. Three days after the induction, culture supernatants were harvested, clarified by centrifugation at 6,000 × g for 30 min at 4°C and filtered through a 0.45 μm filter. Filtered supernatants were concentrated 5-fold using QuixStand Benchtop System incorporated with a 50,000 NMWC Hollow Fiber Cartridge (Model UFP-50-C-4MA). HIV-1 VLP in concentrated supernatant was pelleted by ultracentrifuge through 20% sucrose cushion, resuspended in PBS, and stored in aliquots at -80°C until further use (see below). During 11 day's fed batch culture, small aliquot of cell suspension was collected every 24 h. The number and viability of cells were counted by trypan blue exclusion assay. The amount of HIV-1 VLP, as measured by HIV-1 gp120 and gag p55, was determined (see below). Soluble gp120 in concentrated supernatants were purified by Ni-NTA column according to manufacturer’s instruction (Invitrogen). The amount and the purity of proteins was quantified by BCA assay (Pierce) and determined by 10% SDS/PAGE followed by Coomassie blue staining.

Western blot analysis

To test HIV-1 envelope and gag protein expression, S2 clones were induced with 5μM of CdCl₂ for 3 days. Culture supernatants were harvested, precipitated
by TCA and dissolved in PBS. Samples were separated through 10% SDS/PAGE and transferred onto PVDF membranes (Millipore). The membranes were blocked in a solution of Tris-buffered saline containing 5% nonfat dry milk and 0.05% Tween 20 and subsequently probed with an anti-gag p24 antibody and with indicated antibodies specific for HIV-1 gp120 and gp41. Antigens were visualized with an AP-conjugated anti-mouse IgG antibody according to manufacturer’s instruction (Promega).

To test the incorporation of HIV-1 envelope and gag proteins into VLP, the VLP-containing supernatants were harvested, clarified by centrifugation at 6,000 × g for 30 min at 4°C and filtered through a 0.45 μm filter, loaded onto 20% sucrose cushion and ultra-centrifuged at 20,000 rpm for 2.5 h at 4°C in a SW28 rotor (Beckman Coulter, Fullerton, CA). The pellets were resuspended and fractionated through a 25 – 65 % sucrose density gradient at 25,000 rpm for 16 h at 4°C in a SW41 rotor (Beckman Coulter). Twelve fractions (1 ml each) were collected from top to bottom of the gradient and the density of each fraction was measured. The samples were then TCA precipitated, separated by 10% SDS-PAGE and transferred onto PVDF membranes. The presence of HIV-1 gag and envelope proteins in unfractinated and fractionated samples were detected as described above.

To test mammalian expression of plasmids CMV/R-gp120 (Consensus B and C) and CMV/R-Gag(Pr55), 293 T cells were transiently transfected with plasmids encoding soluble consensus B and C HIV-1 gp120 envelope proteins or gag(Pr55). Two days after transfection, culture supernatants were harvested,
pelleted by ultra-centrifugation, separated by 10% SDS-PAGE and transferred onto PVDF membranes. The presence of HIV-1 gp120 or gag(Pr55) proteins was detected by indicated antibodies against gp120 or gag p24.

To quantify the amount of gag p55 in supernatants produced by S2 clones in fed batch culture, western blot and densitometry analyses were performed as reported by Lynch et al. (27). Briefly, culture supernatants were collected daily during 11 day’s fed batch culture (see above) and loaded onto 4-12% Bis-Tis gel (Invitrogen) along with a serially 2-fold diluted p24 standards (starting at 40 ng) (Aalto BioReagents) and transferred onto PVDF membranes. The membranes were blocked in a solution of TBST containing 5% nonfat dry milk and subsequently probed with an anti-gag p24 antibody. Antigens were visualized with an HRP-conjugated anti-mouse IgG antibody (MultiSciences) and EZ-ECL substrate (Thermo). Gag concentrations were estimated by comparing calculated densities of the p55 bands in experimental samples and p24 standards using Quantity One software (Bio-Rad). Consistent with what was previously reported (27), we also found that HIV-1 VLP concentrations determined by calculating densities of the p55 band on western blots is a more reliable indicator of actual VLP concentration in the sample than using p24 ELISA quantification (Song et al. data not shown).

ELISA

To estimate the amount of HIV-1 envelope incorporation into HIV-1 VLP, 96-well EIA/RIA flat bottom plate (Costar) were coated overnight with 1μg/ml anti-HIV-1 gp120 C5 capture antibody. The plates were blocked with PBS containing
5% BSA for 1 h at 37 °C. The VLP containing culture supernatants, above concentrated VLP-containing samples, or a serial diluted purified gp120 (serve as standards) in assay diluent (10% BSA, 0.5% Triton X-100 in PBS) were added onto anti-gp120 antibody-coated 96 well plates and incubated at 37°C for 2 h. The plates were then washed 5 times with PBST buffer (0.05% Tween 20 in PBS). Anti-gp120 antibody (Cat. #4301) at 1:2,000 dilution was then added and incubated for another 1 h. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Chemicon) at 1:5,000 dilution was added. Colorimetric analysis was performed using the TMB Substrate Kit (Pierce) and absorbance was read at 450 nm by a spectrophotometer (BioTek Instruments, Winooski, VT, USA).

To determine the antigenicity of HIV-1 VLP, a similar sandwich ELISA was performed with 10-fold diluted human anti-gp120 and gp41 antibodies (b12, VRC01, 2G12, PG16 and 4E10) and control antibody (100F4) followed by HRP-conjugated goat anti-human Ig(H+L) (IgM+IgG+IgA, SBA) at 1:2,000 dilution as a secondary antibody. Colorimetric analysis was performed as described above.

To test total IgG, IgG1 and IgG2a antibody responses against consensus B and consensus C gp120, 96-well EIA/RIA flat bottom plates were coated with 100 ng/well of the respective consensus B or consensus C gp120 proteins at 4°C overnight. Plates were blocked with PBS containing 5% BSA at 37°C for 1 h and incubated with serial diluted mouse serum samples or pooled human plasmas at 37°C for 2 h. Plates were washed 5 times with PBST buffer and incubated with HRP-conjugated anti-mouse IgG (1:5,000) or anti-human IgG (1:2,000), anti-mouse IgG1 (1:500) or anti-mouse IgG2a (1:500) antibodies, respectively, at
37°C for 30 min. The wells were washed 5 times with PBST buffer. Colorimetric analysis was performed as described above. End-point titers were determined as the last reciprocal serial serum or plasma dilution at which the absorption at 450 nm was greater than the mean plus 2 SD of the background signals generated by pre-immune serum samples.

**Characterization of glycosylation of HIV-1 envelope proteins produced by S2 clones**

The glycosylation pattern of HIV-1 gp120 produced by stably transfected S2 cells was measured using DIG Glycan Differentiation Kit according to the manufacturer’s instruction (cat. #11210238001, Roche). Briefly, purified HIV-1 gp120 produced by stably transfected S2 cells along with internal controls supplied with the Kit were separated on 10% SDS/PAGE and transferred onto PVDF membranes. The membranes were blocked in 20 ml blocking solution supplied by the Kit for 30 min. After washed twice with TBS, membranes were incubated with digoxigenin-labeled lectins: GNA (galanthus nivalis agglutinin), SNA (sambucus nigra agglutinin), DSA (datura stramonium agglutinin), MAA (maackia amurensis agglutinin) and PNA (peanut agglutinin), respectively, for 1 h. After washed twice with TBS glycosylation pattern was visualized with an AP-conjugated anti-digoxigenin antibody according to manufacturer’s instruction.

To deglycosylate envelop protein produced by S2 cells, 1μg of soluble gp120, HIV-1 VLP- or wild type HIV-1-containing culture supernatants equivalent to 1μg of total proteins were denatured by heating at 100°C for 10 min in the presence of 0.5% SDS and 40 mM DTT. After cooling, 1μl PNGase F or Endo-H...
(New England Biolabs, Ipswich, MA) was added and the mixture was incubated in 1 × G7 or G5 reaction buffers (New England Biolabs) at 37°C for 1 h. Subsequently, samples were subjected to protein gel electrophoresis and Western blot analyses detected by anti-HIV-1 gp120 antibody (#4301) as described above.

Cryo-EM tomography

To purify HIV-1 VLP for cryo-EM study, culture supernatants produced by stably transfected S2 clones were harvested and clarified by low speed centrifuge at 6,000 × g for 30 min at 4°C, passed through the 0.45 μm filter (FISHER), loaded on to 20% sucrose cushion and ultra-centrifuged at 25,000 rpm for 2 h using SW28 rotor. The pellets were resuspended with PBS and loaded onto 25%-65% linear sucrose gradient and ultra-centrifuged at 25,000 rpm for 16 h (SW41 rotor). VLP-containing fractions were pooled and pelleted by ultracentrifugation at 25,000 rpm for 2 h (SW41 rotor). Pellets were resuspended with PBS and loaded onto 30% and 45% non-linear sucrose gradient and ultra-centrifuged at 110,000 × g for 3 h (MLS-50 rotor). Two fuzzy bands, one close to the top of sucrose gradient (upper band) and the other at the sucrose interface (lower band), were collected, diluted in PBS and passed through 0.2 μm low protein binding and non-pyrogenic syringe filter (cat. #PN4612, PALL). The samples were then pelleted by ultracentrifuge at 110,000 × g for 2 h (MLS-50 rotor), resuspended in 20 μl of PBS and stored at -80°C.

To observe VLP by cryo-EM tomography, 3.5 μl of upper and lower band samples were loaded on a 300 mesh R2/1 Quantifoil holey grid (Quantifoil Micro...
Tools, GmbH, Jena, Germany) for 1 minute, blotted for 4s with a filter paper, and rapidly vitrified into liquid ethane using an FEI vitrobot Mark IV. The grid was transferred to a Gatan 626 cryoholder (Gatan, Pleasanton, CA) and examined under low dose conditions on an FEI Tecnai 20 electron microscope operated at 200 kV. Micrographs were recorded on a Gatan Ultrascans 1,000 charge-coupled device camera in an FEI Tecnai F20 electron microscope operated at 200 kV with a magnification of 38,000 x and a defocus of ~2.5 μm. Cryo-EM tomography was performed at an FEI 300 kV Titan Krios cryo-EM equipped with a Gatan UltraScan4000 (model 895) 4K x 4K pixels CCD. Single axis Tilt series were collected at 47,000x magnification using 2K x 2K CCD mode around a single axis at a 2° increment between -62° and +60° at a pixel size of 0.38 nm. The defocus was set at -3 to -6 μm and the cumulative dose was about 72 e/Å².

Immunization and sampling

All animal protocols were approved by the Institutional Animal Care and Use Committee at the Institut Pasteur of Shanghai (Approval No. A2011014). Female BALB/c mice (Mus musculus) at the age of 6 to 8 weeks were purchased from Shanghai Institutes of Biological Sciences Animal Center and housed in microisolator cages ventilated under negative pressure with HEPA-filtered air and a 12/12-h light dark cycle. For the immunization, mice were randomly divided into 2 groups. Group one was intramuscularly (i.m.) injected of both hind legs with total 200 μl PBS (pH 7.4). Group two was i.m. primed twice with 150 μg of three plasmids encoding consensus B and C HIV-1 gp120 proteins and rev-independent HIV-1 gag (50μg each) and then subcutaneously (s.c.) boosted.
twice with a mixture of HIV-1 VLP (consensus B and C) equivalent to 5 μg gp120 plus 5 μg phosphorothioate CpG oligodeoxynucleotides (CpG-ODN 1826 5'-TCC ATG ACG TTC CTG ACG TT-3') (18). HIV-1 VLP used for boost contained a mixture of Gag-alone particles vs. Env-Gag particles, which was likely in a 1 to 1 ratio, because the optical density of two fuzzy bands (upper band containing Gag-alone particles vs. lower band containing Env-Gag particles) collected after the ultracentrifugation in 30% and 45% non-linear sucrose gradient were vary similar (see above). The DNA prime was done on day 0 and 28 and the HIV-1 VLP/CpG boost was done on day 56 and 84. Seven days before prime and 10 days after boost, blood samples were collected by retro-orbital plexus puncture. Serum samples were collected, heat inactivated at 56°C and stored in aliquots at -20°C. Ten days after the boost spleens were harvested and splenocytes were isolated by Ficoll-Hiquac gradient centrifugation, depleted of erythrocytes by treatment with NH₄Cl (0.1 M, pH7.4). Cells were then washed with PBS and resuspended in complete RPMI 1640 medium [i.e. RPMI 1640 supplemented with 10% inactivated FBS, 100 U/ml penicillin, 100 U/ml streptomycin and 100 mg/L L-glutamine] for intracellular cytokine staining (see below).

**HIV-1 and 10A1 pseudotypes and a single-cycle infectivity assay**

HIV-1 and 10A1 pseudotypes were generated and a single-cycle infectivity assay was performed as described before (32, 54).

**FACS analysis**

To study cell surface expression of HIV-1 envelope, chronically HIV-1 infected CEMss-CCR5 cells (see above) were incubated with the post-immune
sera, sera from PBS control mice or pooled HIV-1 infected patient sera for 45 min on ice. Cells were then washed twice with FACS buffer (PBS containing 1% BSA and 0.02% NaN3) and stained with PE-conjugated anti-mouse or anti-human IgG antibody (Sigma) for another 45 min on ice. Cells were then washed twice with FACS buffer and fixed with 1% formaldehyde in 0.5 ml of FACS buffer. FACS analysis was performed on a BD LSRII flow cytometer (BD Biosciences).

**ADCC assay**

The rapid fluorometric ADCC assay was performed as previously described (14, 45). Briefly, 5,000 HIV-1-infected CEMss-CCR5 target cells were doubly labeled with 5 μM PKH-26 (Sigma-Aldrich) and 0.5 μM CFSE (Molecular Probes). Labeled target cells were resuspended in RPMI 1640 medium containing 10% FBS and incubated with pre- and post-immune sera from PBS control and immunized mice, naïve mice sera (negative control) or pooled HIV-1 patient sera (positive control) at the final dilution of 1:50 in a 96-well micro-plate for 30 min at room temperature. Splenocytes (effector cells) from naïve mice were added at a 50:1 effector/target cell (E:T) ratio. The plates were centrifuged for 5 min at 400 × g to promote cell-to-cell interactions and then incubated for 4 h at 37 °C in 5% CO2. Cells were washed twice with PBS, fixed in 3.7% paraformaldehyde–PBS (v/v) for flow cytometry. Fifty thousand non-gated events in duplicate wells were acquired within 18 h by using BD LSRII flow cytometer. Data were analyzed using FlowJo (Tree Star Inc., USA). The percent ADCC killing was determined by back-gating on the PKH-26\textsuperscript{high} population of target cells that lost the CFSE viability dye and subtracted the nonspecific effect by pre-immune sera. Non-stained and
single-stained targets were included in every experiment to compensate for single-stained CFSE and PKH-26 emission.

**ADCVI assay**

Target and effector cells used for the ADCVI assay were the same HIV-1-infected CEMss-CCR5 cells and naïve splenocytes used for ADCC (see above). Target cells (5,000) were washed to remove cell-free virus. Effector cells (naïve splenocytes) were added to target cells at an E:T ratio of 20:1. Pre- and post-immune sera from PBS control and immunized mice, naïve mice sera (negative control) or pooled HIV-1 infected patient sera (positive control) were added to target and effector cells to achieve a final dilution of 1:50. Control wells lacking sera but containing effector cells and viral replication control wells lacking both sera and effector cells were included. Two days later, supernatants were collected, and gag p24 was measured by ELISA (Zeptometrix). The percent inhibition due to ADCVI was calculated relative to pooled pre-immune mice sera as follows: percent inhibition = 100[1 – (p24post)/(p24pre)], where (p24post) and (p24pre) are concentrations of p24 in supernatant fluid from wells containing a source of post- or pre-immune sera, respectively. Individual serum samples were assayed in triplicate and in two separate assays. In general, values from the two independent assays were in close agreement with each other.

**Intracellular cytokine staining**

2×10^6 splenocytes from immunized and control mice were seeded into complete RPMI 1640 and stimulated with two mixtures of peptides (2μg/ml each peptide) as previously described (30), one mixture containing peptides derived...
from HIV-1 envelope protein and the other from gag protein, as well as 5 μg of anti-mouse CD28 and 5 μg of anti-mouse CD49d for 6 h at 37°C and 5% CO₂. Golgi plug (BD Pharmingen) was added during the final 4 h of incubation. Cells were then stained with anti-mouse CD16/32 (Fc block) antibody followed by surface staining with PerCP-conjugated anti-CD4 and APC-conjugated anti-CD8 antibodies. Cells were then fixed, permeabilized with cytofix cytoperm (BD Pharmingen) and stained with FITC-conjugated anti-IFNγ and PE-Cy7-conjugated anti-TNFα. 1 × 10⁶ cells per sample were acquired on a BD LSRII flow cytometer and FACS data were analyzed using FlowJo software.

**Statistics**

Analyses were performed with GraphPad Prism v 5.0. Unpaired t test was used to compare two data sets. Correlation between the ADCC and ADCVI was assessed by the exact Spearman rank correlation method. All P values are two-tailed.
Results

HIV-1 envelope and gag proteins were properly produced, processed and incorporated into VLP

To generate stably transfected S2 clones that produce high amount of HIV-1 VLP, we co-transfected Drosophila S2 cells with plasmids pAC-Gag(Pr55), pMT/BiP-gp160 (consensus B or C), pMT-Rev and pCoBlast, in which genes encoding HIV-1 envelope and rev proteins were driven by an inducible pMT promoter; while gene encoding HIV-1 gag(Pr55) was driven by a constitutive pAC promoter (Fig. 1A). After the stable transfectants were established, limiting dilution assay was performed to generate stably transfected S2 clones. Fig. 1B shows that in supernatants from S2 clone VB2, but not from parental S2 cells, HIV-1 gag p55 was readily detected with or without induction; whereas without induction a faint gp120 was detected, while after induction the expression of gp120 increased dramatically. Fig. 1C shows that after induction both gp41 and gp160 of HIV-1 envelope proteins were detected by antibody 4E10, but only gp120 was detected by antibody VRC01, indicating that although precursor HIV-1 envelope gp160 were properly cleaved into gp120 and gp41, because the high level of expression of HIV-1 gp160 in stable S2 transfectants, only over 60% of gp160 was cleaved by cellular endopeptidase(s) during their biogenesis through Golgi apparatus. Similar studies on the induction of envelope protein and cleavage of gp160 into gp120 and gp41 were also observed in S2 clone VC1 (that expressed consensus C HIV-1 envelope protein) (Yang et al. data not shown).
To test the incorporation of envelope and gag proteins into HIV-1 VLP, S2 clone VB2 was induced with CdCl₂ and supernatants were harvested, concentrated, and then fractionated through a 25-65% sucrose gradient. The envelope and gag proteins in each fraction were detected with a mixture of two mouse antibodies against gp120 and gag p24. As a control, supernatants from S2 cells stably transfected with pAC-Gag(Pr55) alone were also harvested, concentrated and fractionated through the same sucrose gradient. As shown in Fig. 1D gag proteins derived from S2 cells transfected with pAC-Gag(Pr55) alone were peaked in the fractions with buoyant density between 1.10 and 1.14; whereas both gp160 and gp120 and gag proteins derived from S2 clone co-transfected with pAC-Gag(Pr55) and pMT/Bip-gp160 were peaked in the fractions with buoyant density between 1.15 and 1.19 (Fig. 1E), indicating likely both uncleaved gp160 and cleaved gp120/gp41 were incorporated into the VLP and the difference in 160 kD gp160 band shown in Fig. 1E, but not in Fig. 1C was because two different anti-HIV-1 gp120 antibodies (VRC01 and #4301) were used to detect HIV-1 glycoproteins. In western blot antibody VRC01 only reacts with gp120; whereas antibody #4301 reacts with both gp120 and gp160. Similar study was performed in the stable S2 clone VC1 with similar results (Yang et al. data not shown). Thus, taken together, these results clearly indicate that in the S2 clones HIV-1 envelope proteins are properly induced, cleaved and both envelope and gag are incorporated into HIV-1 VLP.

**Glycosylation of envelope proteins produced by S2 clones**

To test glycosylation of envelope proteins on HIV-1 VLP produced by S2...
clone VB2, we first performed deglycosylation assays with N-endoglycosidase PNGase F and Endo H. For a comparison, glycosylation of soluble gp120 produced by S2 clone B5 (that expressed consensus B gp120) and wild type HIV-1 (Bru-3) were also tested. PNGase F removes all types of N-linked oligosaccharides from glycoproteins, whereas Endo H cleaves the chitobiose core of high-mannose and hybrid oligosaccharides from N-linked glycoproteins.

Fig. 2A shows that without PNGase F or Endo H treatment, 120 KD band was detected by anti-gp120 antibody in soluble gp120 as well as in HIV-1 VLP; whereas with PNGase F treatment, gp120 bands of soluble gp120 and HIV-1 VLP were reduced to 70 KD band; whereas with Endo H treatment gp120 bands of soluble gp120 and HIV-1 VLP were reduced to about 74 KD and 85 KD bands, respectively. Similar, but not identical, deglycosylation pattern was seen in envelope proteins of wild-type HIV-1 virions with PNGase F or Endo H treatment (Fig. 2A). Thus, these results not only revealed that soluble gp120 and envelope protein on HIV-1 VLP produced by S2 clones are N-link-glycosylated, but also highlighted some difference in N-linked glycosylation between soluble gp120 and envelope proteins on HIV-1 VLP even though these envelope glycoproteins were both produced by clones derived from the same S2 cells.

To further test glycosylation patterns, soluble gp120 produced by S2 clone B5 were probed with lectins GNA, SNA, MAA, PNA and DSA that recognize different sugar moieties (see Materials and Methods). As shown in Fig. 2B large amount of gp120 produced by the S2 clone was detected by GNA and small amount was detected by PNA and DSA, but not by SNA and MAA, indicating that
likely, on a given HIV-1 envelope glycoprotein produced by S2 clones there are more high-mannose glycans and less hybrid and complex forms.

**Quantification of HIV-1 VLP produced by S2 clones**

To test the cell growth and HIV-1 VLP production by S2 clones, S2 clones were grown in fed batch culture using the Wave bioreactor for a total of 11 days. Eight days after the initial fed batch culture CdCl₂ was added to induce HIV-1 envelope expression (see Materials and Methods). Fig. 3A shows the growth of a representative S2 clone VB2 in fed batch culture. Starting with 300 ml of seed cell suspension, 300, 200 and 200 ml of fresh culture medium were added on day 5, 7 and 8, respectively, and cell density grew from 2 x 10⁶ cells per ml on day 0 to 2.2 x 10⁷ cells per ml on day 11. By so doing, the total viable cell numbers grew about 37 folds from 6 x 10⁸ cells on day 0 to 2.2 x 10¹⁰ cells on day 11 (Fig. 3B). Fig. 3C shows that while the amount of gag p55 in the supernatants grew steadily and reached 9.5 μg per ml on day 11; the amount of gp120 in the supernatants before the CdCl₂ induction were very low, but after the induction the amount of gp120 increased dramatically and reached 4.2 μg per ml on day 9 and 7.5 μg per ml on day 11. Similar cell growth and HIV-1 VLP production were also observed in another S2 clone VC1 (Song et al. data not shown).

**Morphology and spike number on HIV-1 VLP produced by S2 clones**

To assess the morphology of the HIV-1 VLP produced by S2 clones, HIV-1 VLP in culture supernatants were concentrated and purified (see Materials and Methods) and the morphology and spikes on the surface of purified HIV-1 VLP were analyzed by cryo-EM and electron tomography. Fig. 4A and 4B show the
particles obtained from upper and lower bands of representative HIV-1 VLP samples derived from S2 clone VB2 (see Materials and Methods for the detail). Particles obtained from both upper and lower bands were intact and round. The diameters of particles ranged from 96 nm to 185 nm with an average diameter of 125.7 ±23.2 nm (Table 1), which is slightly larger but comparable with those of native SIV and HIV-1 virions we measured in the similar cryoEM tomograms (109 ± 14 and 110 ± 8 nm for SIV and HIV-1 virus respectively) (62). It is also consistent with the measurements of SIV and HIV-1 virions in the negative stain electron tomograms (61). At this time, we do not really know what accounts for the heterogeneity in the size of the VLPs. But it may be due to the lack of HIV-1 protease in VLPs to process immature p55 Gag. Interestingly, particles collected from the upper band did not exhibit any spikes on the HIV-1 VLP surface (Fig. 4A); whereas on the surface of particles collected from lower band, envelope spikes were readily observed (Fig. 4B). Electron tomography with selected 12 particles collected from the lower band showed average 17 ± 2 spikes per particle (ranging from 13 to 20 spikes) (Fig. 4C and Table 1). Furthermore, like those observed on the surface of SIV or HIV-1 virions (62), no discrete periodic distances between spikes on the surface of HIV-1 VLP were observed (Fig. 4D).

**Antigenicity of HIV-1 envelope glycoproteins produced by S2 clones**

To test antigenicity of HIV-1 envelope glycoproteins produced by stably transfected S2 clones, we performed three sets of experiments with a panel of broadly neutralizing antibodies 2G12, b12, VRC01, PG16 and 4E10 (6, 7, 52, 55, 60, 63) as well as control antibody 100F4 antibody (53). The antigenicity results of
HIV-1 envelope glycoproteins were summarized in Table 2. In the first experiment we compared antigenicity of HIV-1 envelope glycoproteins on cell surface between human kidney cell line 293 T transiently transfected with CMV/R-gp160 (consensus B) and S2 clone VB2 that expressed the same gp160 (consensus B). Supplementary Fig. 1 shows that on the surface of transduced 293 T cells HIV-1 envelope glycoproteins were recognized by all broadly neutralizing antibodies 2G12, b12, VRC01, PG16 and 4E10, but not by control antibody 100F4; whereas on the surface of S2 clone HIV-1 envelope glycoproteins were recognized by antibodies 2G12, b12, VRC01 and 4E10, but not by PG16 and control antibody 100F4.

In the second experiment, we measured antigenicity of HIV-1 VLP by western blot analysis. Supplementary Fig. 2 shows that HIV-1 VLP was only recognized by broadly neutralizing antibodies VRC01, 2G12 and 4E10, but not by b12, PG16 and control antibody 100F4.

In the third experiment, we measured antigenicity of HIV-1 VLP by sandwich ELISA using serially diluted antibodies (see Materials and Methods). Supplementary Fig. 3 shows that HIV-1 VLP was only bound by broadly neutralizing antibodies 2G12, b12, VRC01 in a dose-dependent manner, but not by PG16 and 4E10 nor by control antibody 100F4. Thus, taken together we conclude that except for antigenic epitope PG16, all other broadly neutralizing antigenic epitopes 2G12, b12, VRC01 and 4E10 tested are preserved on spikes of HIV-1 VLP produced by S2 clones.

**ELISA-binding antibody responses elicited by DNA-VLP prime-boost**
In our initial studies on immunogenicity of HIV-1 VLP, we found that HIV-1 VLP produced by S2 clones was immunogenic even without a supplement of any adjuvant. However, priming with DNA and boosting with HIV-1 VLP enhanced immunogenicity (Yang et al. data not shown), which was similar to what was reported by Buonaguro et al. (5). In addition, we demonstrated that heterologous DNA-VLP prime-boost elicited superior neutralizing antibody response and protection in mice against HPAI H5N1 virus than homologous DNA-DNA or VLP-VLP vaccination (10). Therefore, in the present study, immunogenicity of HIV-1 VLP produced by stably transfected S2 clones was evaluated in the heterologous DNA-VLP prime-boost setting. Specifically, BALB/c mice were i.m. primed twice with three plasmids CMV/R-gp120 (consensus B and C) and CMV/R-Gag (Pr55) and s.c. boosted twice with a mixture of HIV-1 VLP expressing consensus B and C envelope proteins in the presence of CpG. BALB/c mice injected with PBS were used as controls. Serum samples were collected before and after immunization and splenocytes were harvested after sacrifice. No animals showed any signs of toxicity, and all remained healthy up to the end of the immunization protocol.

To evaluate HIV-1 envelope-specific serum antibody titers, ELISA was performed on microwell plates coated with consensus B and C gp120, respectively. For the comparison, pooled HIV-1 patient plasmas were used. The plasmas were pooled by 8 plasma samples selected from over 800 samples of HIV-1 B’C recombinant virus-infected individuals in China. In our unpublished study these 8 individual plasmas broadly neutralized various subtypes of HIV-1
strains (Yang et al. data not shown). Fig. 5A and B show that both mouse immune sera and pooled human plasmas exhibited very high end-point titers with average titers of $1.77 \times 10^7$ in mouse immune sera and $2.37 \times 10^7$ in human plasmas against consensus B gp120 and average titers of $5.43 \times 10^6$ in mouse immune sera and $3.67 \times 10^7$ in human plasmas against consensus C gp120. Fig. 5C and D show that high titers of specific serum IgG1 against consensus B or C gp120 were elicited in the immunized group. At 1 to $10^4$ dilutions, OD values ranging from 0.7 to 1.7 were detected against consensus B gp120 and from 0.8 to 1.6 against consensus C in the immunized group. Compared to OD values detected in PBS control group the difference is very significant ($P < 0.0001$ for consensus B gp120; $P < 0.0001$ for consensus C gp120). Fig. 5E and F show that high titers of specific serum IgG2a against consensus B or C gp120 were elicited in the immunized group. At 1:10^4 dilutions, OD values ranging from 0.1 to 0.8 were detected against consensus B gp120 and from 0.1 to 0.7 against consensus C in the immunized group. Compared to OD values detected in PBS control group the difference is statistically significant ($P = 0.0371$ for consensus B gp120; $P = 0.0111$ for consensus C gp120). Thus we conclude that heterologous DNA-VLP prime-boost elicits very high titers of ELISA-binding total IgG, IgG1 and IgG2a responses against both consensus B and C envelope proteins.

Neutralizing antibody responses elicited by DNA-VLP prime-boost

To test neutralization activity of the immune sera, HIV-1 pseudotype-based neutralization (PN) assay was performed against a panel of HIV-1 pseudotypes as well as 10A1 control. The retroviral envelope 10A1 recognizes either Ram-1 or
Glvr-1 as a receptor for cell entry (32) and was used here as negative control. The panel of HIV-1 pseudotypes consists of HIV-1 envelopes derived from clade A (Q168), clade B (consensus B, AD8, HXBc2 and Yu2), clade B’ (CNE11), clade C (consensus C) and CRF01_AE (CNE3). Fig. 6A shows neutralization activity of immune sera at 1:50 dilutions against the pseudotype panel. As expected, compared with pseudotypes alone, immune sera from all 6 immunized mice exhibited no neutralization activity against control 10A1 pseudotype. Compared with neutralization activity against control 10A1 pseudotype, there was no significant neutralization activity of the immune sera against AD8 pseudotype. In contrast, the immune sera from 6 immunized mice exhibited statistically significant neutralization activity against Q168, consensus B, HXBc2, Yu2, consensus C, CNE3 and CNE11 pseudotypes. Among them, on average there were 60% and 40% neutralization activity against autologous consensus B and C pseudotypes respectively, and 51, 42, 41, 36 and 21% against heterologous CNE11, HXBc2, CNE3, Yu2 and Q168 pseudotypes, respectively.

To further test neutralization activity, the immune sera of individual immunized mice were titrated against HIV-1 pseudotypes expressing consensus B, consensus C, CNE11 and CNE3 envelope proteins (Fig. 6B). On average at 1:20 dilution 70% neutralization activity against consensus B, 54% neutralization activity against consensus C, 60% neutralization activity against CNE11 and 55% neutralization activity against CNE3 were detected and at 1:60 dilution neutralization activity dropped to 50% neutralization activity against consensus B, 20% neutralization activity against consensus C, 40% neutralization activity...
against CNE11 and 35% neutralization activity against CNE3. The pre-immune sera from immunized mice did not show any neutralization activity. With the same pooled human plasmas, we also titrated their neutralizing antibody activity against HIV-1 pseudotypes expressing consensus B, consensus C, CNE3 and CNE11. As shown in Supplementary Fig. 4, these highly selected, pooled human plasmas exhibited comparable to or even higher neutralizing antibody activity than mouse immune sera shown in Fig. 6 B. Thus, we conclude that DNA-VLP prime-boost elicits neutralizing antibody responses against a number of autologous and heterologous envelope proteins. But their potency and breadth still need to be improved.

**ADCC and ADCVI responses elicited by DNA-VLP prime-boost**

To test whether immune sera elicited with DNA-VLP prime-boost could mediate antibody-dependent cell-mediated viral inhibition (ADCVI), we first infected CEMss-CCR5 cells with HIV-1 AD8 strain and 15 days later measured virus replication by HIV-1 gag p24 ELISA and stained cell surface expression of HIV-1 envelope protein using pooled HIV-1 patient sera followed by FACS. We found that HIV-1 replicated well in infected CEMss-CCR5 cells (Yang et al. data not shown) and HIV-1 envelope proteins were expressed on the surface of infected cells (the right panel of Fig. 7A). We then stained cell surface expression of HIV-1 envelope protein using sera from PBS control mice and immune sera elicited with DNA-VLP prime-boost. The left and middle panels of Fig. 7A show that immune sera elicited with DNA-VLP prime-boost mice, but not from PBS control, recognized HIV-1 envelope proteins on the surface of infected cells.
We next investigated whether immune sera could mediate ADCC using AD8-infected cells as target cells and naïve mouse splenocytes as effector cells. Fig. 7B shows that at a serum dilution of 1:50, ADCC activity ranging from 5 to 12% was observed in immune sera from all 6 immunized mice; whereas no ADCC activity was observed in sera from all 6 PBS control mice. The difference was also highly statistically significant ($P = 0.0002$). The experiment was repeated twice with similar results.

ADCVI of immune sera was also investigated using the same AD8-infected cells and naïve mouse splenocytes as target and effector cells, respectively. Fig. 7C shows that at a serum dilution of 1:50, ADCVI activity ranging from 15% to 40% was observed in immune sera from all 6 immunized mice; whereas no ADCVI activity was observed in sera from all 6 PBS control mice. The difference was highly statistically significant ($P = 0.006$). The experiment was repeated twice with similar results. Fig. 7D shows that there was a positive correlate between % of killing by ADCC and % of inhibition by ADCVI ($r = 0.9161$) when sera from all immunized and PBS control mice were analyzed.

**CD8 T cell responses elicited by DNA-VLP prime-boost**

To test whether DNA-VLP prime-boost could elicit HIV-1 envelope and gag-specific T cell responses, splenocytes from DNA-VLP prime-boost and PBS control mice were assayed against envelope or gag peptide mixtures using intracellular cytokine staining for IFNγ and TNFα. Fig. 8A shows that compared to CD8 T cells from PBS control mice, CD8 T cells from DNA-VLP prime-boost mice exhibited statistically significant peptide-specific responses against envelope
peptides. On average, 0.35% of CD8 T cells secreted IFNγ and 0.39% of CD8 T cells secreted TNFα. In contrast, no CD8 T cell response against envelope peptides was observed in splenocytes from all 6 PBS control mice. The differences were highly statistically significant ($P < 0.0001$ for both IFNγ and TNFα). Fig. 8B shows that compared to CD8 T cells from PBS control mice, CD8 T cells from DNA-VLP prime-boost mice exhibited statistically significant peptide-specific responses against gag peptides. On average, 0.06% of CD8 T cells secreted IFNγ and 0.07% of CD8 T cells secreted TNFα. In contrast, no CD8 T cell response against gag peptides was observed in splenocytes from all 6 PBS control mice. The differences were highly statistically significant ($P = 0.009$ for IFNγ, $P = 0.0226$ for TNFα). Thus, we conclude that the DNA-VLP prime-boost elicits both HIV-1 envelope and gag-specific CD8 T cell responses.
Discussion

In the present study, we developed stably transfected Drosophila S2 cell clones to produce HIV-1 VLP and demonstrated that in one liter of culture supernatant the amount of HIV-1 VLP produced by S2 cell clones is equivalent to 7.5 mg of gp120 and 9.5 mg of gag p55 (Fig. 3C), which is comparable to that produced by insect cells infected with RB (5, 41) and much higher than those produced by mammalian cells (16, 17, 30, 36, 42). The high HIV-1 VLP production is mainly due to the fact that stably transfected S2 clones could grow into much higher viable cell density than mammalian cells. In our present study, stably transfected S2 clones in the fed batch culture using Wave bioreactor grew to 2.2 x 10^7 cells per ml without significantly compromising cell viability (Fig. 3A and 3B). In our study with other S2 clones, we found that using Wave bioreactor stably transfected S2 clones in perfusion culture could grow to 1 x 10^8 cells per ml without compromising cell viability (Wang et al. Mol. Biotech. In press).

Therefore, there is still large room to improve HIV-1 VLP production by our stably transfected S2 clones. In addition, unlike HIV-1 VLP produced by insect cells infected with RB, envelope proteins in HIV-1 VLP produced by stably transfected S2 clones were properly cleaved into gp120 and gp41 (Fig. 1C). Moreover, unlike HIV-1 VLP produced by insect cells infected with RB, culture supernatants produced by stably transfected S2 clones are not contaminated with recombinant viruses. Thus, compared to current expression systems, stably transfected S2 clones truly have several distinct advantages for HIV-1 VLP production.

In the present study, we also demonstrated that mice primed with plasmids
expressing HIV-1 gp120 and gag and boosted with HIV-1 VLP produced by stably transduced S2 clones in the presence of CpG exhibited both anti-envelope antibody responses (see Fig. 5, 6 and 7) and envelope and gag-specific CD8 T cell responses (see Fig. 8). Anti-HIV-1 envelope antibody responses include ELISA-binding, neutralizing, ADCC and ADCVI. Percentage of killing by ADCC and percentage of inhibition by ADCVI are positively correlated (Fig. 7D). The protective role of envelope and gag-specific CD8 T cells in the control of SIV replication has been inferred by several studies in macaques (29, 43, 48). The protective role of neutralizing antibodies has also been demonstrated in SHIV-infected macaque models through passive immunization (1, 28, 49). However the in vivo mechanism of protection through passive immunization of antibody b12 was in part, FcγR mediated (19). Indeed, vaccine studies in macaques (2, 15, 20, 56) and analyses of sera from HIV-1 infected individuals (13, 46) have shown an inverse correlation between the level of ADCC or ADCVI and virus load. Thus, although exact correlates of protection against HIV-1 is still not fully established, taking into account the correlates of immune protection against other viral pathogens as well as understanding the immune components that control HIV-1 in vivo, the consensus view now is that a highly effective vaccine will need to elicit coordinated antibody and T cell responses (31). Thus, the demonstration that DNA-VLP prime-boost could elicit both antibody and T cell responses in mice and that antibody responses include ELISA binding, neutralizing, ADCC and ADCVI in the present study makes HIV-1 VLP produced by stably transduced S2 clones a promising vaccine component.
Although our present study focused on DNA prime and VLP/CpG boost to evaluate immunogenicity of HIV-1 VLP, in our preliminary study, we compared immunogenicity between DNA prime and VLP/CpG boost versus DNA prime and soluble gp120/CpG boost in mice. We found that although DNA-VLP/CpG elicited higher, but not statistically significant, ELISA-binding antibody and CD8 T cell responses than DNA-gp120/CpG, neutralizing antibody responses elicited by DNA-VLP/CpG vaccination are significantly higher than those elicited by DNA-gp120/CpG (Yang et al. data not shown). We also compared immunogenicity between DNA-DNA, VLP/CpG-VLP/CpG and DNA-VLP/CpG in mice. We found that DNA-VLP/CpG elicited significantly higher ELISA binding and neutralizing antibody responses than those elicited by DNA-DNA and VLP/CpG-VLP/CpG vaccination (Yang et al. data not shown).

In the present study we also show that envelope proteins in HIV-1 VLP produced by stably transfected S2 clones are recognized by several broadly neutralizing antibodies 2G12, b12, VRC01 and 4E10, but not by PG16 (see Table 2 and supplementary Fig. 1, 2 and 3). We found that while antibody 2G12 could bind to envelope proteins on both transfected 293 T cells and S2 cells; antibody PG16 could only bind to envelope proteins on the surface of transfected 293 T cells, but not of transfected S2 cells (Table 2 and Supplementary Fig. 1).

Antibody 2G12 recognizes terminal dimannose (Man\(\alpha 1,2\)Man) moieties on oligomannose glycans (7) and antibody PG16 recognizes a conformational epitope that is dependent on glycosylation at specific variable loop N-linked sites (12, 52). The difference in PG16 recognition of envelope proteins expressed on
the surface of transfected 293 T cells and S2 cells, therefore, is likely due to the heterogeneity in glycosylation. Recently, six new human broadly neutralizing antibodies PGT 125, 126, 127, 128, 130 and 131 that bind specifically to the Man8/9 glycans on gp120 were reported (35, 51). Among them, PGT 128, the broadest of these antibodies, neutralizes over 70% of globally circulating viruses and is, on average, an order of magnitude more potent than PG16 and VRC01 (51). PGT128 was found to bind to two conserved glycans and a short β-strand segment of the gp120 V3 loop (35), therefore it will be interesting to test whether these new antibodies can recognize glycans on envelope proteins produced by stably transfected S2 clones, which was found to be mainly glycosylated in a high mannose form with a few in complex and hybrid (Fig. 2A and B).

Finally, careful analysis of morphology of HIV-1 VLP produced by stably transfected S2 cell clones also points out an inherited shortcoming in the vector system we used in the present study. We found that because in the vector system the gag gene was under a constitutive promoter and the envelope gene was under an inducible promoter, there are two kinds of HIV-1 VLP in supernatants. One kind of HIV-1 VLP produced before cells were induced by CdCl₂ are mainly present in the upper band of non-linear sucrose gradient and do not have spikes on their surface (Fig. 3C and 4A) and have lower buoyant density between 1.10 and 1.14 (Fig. 1D); while the other kind produced after cells were induced by CdCl₂ are present only in the lower band of non-linear sucrose gradient and do have spikes on their surface (Fig. 3C and 4B) and have higher buoyant density between 1.15 and 1.18 (Fig. 1E). Importantly, using cryo-EM
and electron tomography method, we were able to analyze spikes on the surface of a dozen of representative spike-containing HIV-1 VLP and found that average 17 spikes per particle are present on particle surface after the purification (Table 1), which is similar to what was previously reported in wild type HIV-1 particles (62). Furthermore, like those observed on the surface of SIV or HIV-1 virions (62) we also did not find discrete periodic distances between spikes on the surface of HIV-1 VLP (Fig. 4D) as one may have expected if spike placement reflected an underlying geometry of matrix and capsid gag structure. Furthermore, since we did observe that uncleaved gp160 as well as cleaved gp120/gp41 co-migrated with HIV-1 Gag in sucrose gradient (Fig. 1E), likely the spikes on the surface of HIV-1 VLP observed in Fig. 4C represent both uncleaved gp160 as well as cleaved gp120/gp41, which can not be distinguished by the current reconstruction with cryoEM tomography. Therefore, further refinement of vector system to express gag and envelope proteins under both constitutive or both inducible promoters, as well as production, induction and purification protocols are needed to maximize the yield of envelope spike containing HIV-1 VLP.
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monkeys by a vaginally applied monoclonal antibody to HIV-1 gp120. Nat Med 9:343-6.
Table 1. Measurement of purified HIV-1 VLP by cryo-EM and tomography.

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<th>HIV-1 VLP</th>
<th>diameter (nm)</th>
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<td>Mean±SD</td>
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Table 2. Summary of antigenicity of HIV-1 VLP measured by FACS, Western Blot and ELISA.

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**Figure Legends**

**Fig. 1** Expression, cleavage and incorporation of HIV-1 envelope and gag proteins produced by S2 clones. A. Schematic diagrams of plasmids encoding gp160 (consensus B or C), rev, gag p55 proteins and pCoBlast selection marker. pMT/Bip stands for sequences encoding metallothionien promoter and Bip signal peptide; pMT stands for sequence encoding metallothionein promoter and pAC stands for sequence encoding Ac5 promoter. Poly A: poly adenolytion signal. B. Western blot analysis of HIV-1 gag and envelope protein expression with or without the induction of 5 μM CdCl2. C. Western blot analysis of HIV-1 envelope protein expression by anti-gp120 and gp41 antibodies, respective. D. Western blot analysis of HIV-1 gag protein in sucrose gradient-unfractionated and -fractionated samples from HIV-1 gag alone transfected S2 cells. E. Western blot analysis of HIV-1 envelope and gag proteins in sucrose gradient-unfractionated and -fractionated samples from stably transfected S2 clone expressing both HIV-1 envelope and gag proteins.

**Fig. 2** Glycosylation of HIV-1 gp120 or HIV-1 VLP produced by S2 clones. A. Deglycosylation of HIV-1 gp120 protein, HIV-1 VLP and wild type HIV-1 (Bru-3) with the treatment of PNGase F and endo H. B. Probing glycosylation pattern of HIV-1 gp120 with a panel of lectins. GNA stands for galanthus nivalis agglutinin; SNA stands for sambucus nigra agglutinin; DSA stands for datura stramonium agglutinin; MAA stands for maackia amurensis agglutinin; and PNA stands for peanut agglutinin. I.C. stands for an internal control.

**Fig. 3** Growth and HIV-1 VLP production by S2 clones in Wave bioreactor. A.
The viable cell density and the culture volume of S2 clone VB2 during 11 day’s fed batch culture; B. The cell number and the culture viability of S2 clone VB2 during 11 day’s fed batch culture; C. gag p55 and gp120 in supernatants produced by S2 clone VB2 during 11 day’s fed batch culture. Arrow stands for the day in which CdCl2 inducer was added.

**Fig. 4** Representative cryoEM tomographic images of HIV-1 VLP produced by S2 clone VB2. A. Representative computationally derived transverse sections electron micrographs of HIV-1 VLP taken from upper band of non-linear sucrose gradient revealed using cryo-EM. Scale bars = 100 nm. B. Representative computationally derived transverse sections electron micrographs of HIV-1 VLP taken from lower band of non-linear sucrose gradient revealed using cryo-EM. Scale bars = 100 nm. C. Z-stack Computationally derived transverse Z-stack tomographic images of a selected HIV-1 VLP taken from lower band of non-linear sucrose gradient. Red arrows indicate the location of spikes. D. A surface-rendered model of a selected HIV-1 VLP produced by stably transfected S2 clone VB2 with highlighted presumptive ENV spikes (white) derived from electron tomogram.

**Fig. 5** ELISA-binding antibody responses elicited with heterologous DNA-VLP prime-boost. The left panels show the end-point titers of total IgG (panel A), IgG1 (panel C) and IgG2a (panel E) responses specifically against consensus B gp120 and the right panels show the end-point titers of total IgG (panel B), IgG1 (panel D) and IgG2a (panel F) responses specifically against consensus C gp120. In each panel, data represent the mean ± SD from 6 mice immunized with DNA-
VLP prime-boost and 6 mice injected with PBS control. Pooled HIV-1 patient plasmas were combined 8 plasma samples selected from over 800 samples of HIV-1 B‘C recombinant virus-infected individuals in China, which broadly neutralized various subtypes of HIV-1 strains.

Fig. 6 Neutralization activity in individual immune serum samples elicited with heterologous DNA-VLP prime-boost. A. Neutralization activity of immune serum samples at 1:50 dilution against a panel of HIV-1 pseudotypes and 10A1 control in TZM-bl cells. Percentage (%) of neutralization was calculated by [the amount of relative luciferase activity (RLA) in pseudotype alone – the amount of RLA in pseudotype plus immune serum sample]/the amount of RLA in pseudotype alone. Statistic analysis was performed between mean ± SD of a given HIV-1 pseudotype and mean ± SD of 10A1 control. The dash line shows 50% of neutralization; bars stands for means of % neutralization to a given HIV-1 pseudotype; and  stands for P < 0.001. B. Titration of neutralization activity of immune serum samples against HIV-1 pseudotypes consensus B and C, CNE11 and CNE3.

Fig. 7 ADCC and ADCVI responses of individual immune serum samples elicited with heterologous DNA-VLP prime-boost. A. Cell surface expression of HIV-1 envelope proteins in HIV-1 AD8-infected CEMs-CCR5 cells detected by pooled sera from PBS control and immunized mice as well as pooled HIV-1 patient sera. B. HIV-1 AD8-infected CEMs-CCR5 target cells were doubly labeled with PKH-26 and CFSE. The labeled target cells were incubated with splenocytes (effector cells) from a naive BALB/c mouse (E:T = 50:1) and with 1:50 diluted individual
sera samples from PBS control and immunized mice. Eighteen h later, ADCC was determined as described in Materials and Methods. C. HIV-1 AD8-infected CEMss-CCR5 target cells were incubated with splenocytes (effector cells) from naïve BALB/c mice (E:T = 20:1) and with 1:50 diluted individual sera samples from PBS control and immunized mice. Two days later, gag p24 in culture supernatants was determined by ELISA, and virus inhibition was determined as described in Materials and Methods. D. Linear regression analysis between % killing by ADCC and % inhibition by ADCVI among all immunized and control mice.

**Fig. 8** HIV-1 envelope and gag-specific CD8 T cell responses elicited with heterologous DNA-VLP prime-boost. Intracellular cytokine (IFN-γ and TNF-α) staining was performed to analyze the CD8 T cell responses against a panel of HIV-1 envelop-specific peptides (A) and a panel of HIV-1 gag-specific peptides (B). The percentages of activated CD8+ T cells that produce IFN-γ and TNF-α are shown. Splenocytes from mice (n=6 per group) immunized with DNA-VLP prime-boost or mice (n=6 per group) from PBS control were assessed and immune responses were measured 10 days after the final boost.