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Comparison of Immunogenicity between Codon Optimized HIV-1 Thailand Subtype B gp140 and gp145 Vaccines

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Abstract

HIV-1 pandemic posed an unprecedent challenge to the global health and it is believed that an effective vaccine will be the final solution against HIV-1. HIV-1 envelope is the primary immunogen in developing neutralization antibody based HIV vaccine. To define the suitable Env derived immunogen, we systemically compared the immunogenicity of gp140 and gp145 in a DNA vaccination alone and a prime-boost modalities. 2 DNA vaccines and 2 recombinant Tiantan vaccinia vaccines (rTTV) were constructed for vaccination of female Balb/c mice. Elispot assay was used to read out the T cell immunity and ELISA assay was used to quantify antibody immunity. PLL (poly-L-Leucine)-ELISA assay was used in linear antibody epitope mapping. Mice primed with gp145 tended to elicit more Env-specific T cells responses than those primed with gp140, significant difference was observed in DNA immunization alone. The ultimate T cell responses in prime-boost regimen tends to be determined mainly by the priming efficacy. Linear antibody epitope mapping displayed that sera raised by gp145 priming were vigorously reactive to more peptides than that by gp140. Our data demonstrated HIV-1 Thailand B-derived gp145 may raise higher T-cell responses and broader linear peptide-specific antibody responses than gp140 does. However, it remains to be determined that how these observations are relevant to neutralization antibody activities.

Keywords

HIV-1; Vaccine; Envelope; Immunogen design

Introduction

The HIV/AIDS pandemic have globally affected millions of people, an effective vaccine remains elusive to date. Although effective antiretroviral therapy is available in the developed countries, it remains financially unaffordable for the most of HIV-1 infected individuals who

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largely reside in the developing countries. It is thus widely believed that an effective vaccine is the only solution to restrain the global HIV-1 epidemic.

Due to the great challenge to develop an effective vaccine able to induce neutralizing antibodies [1,2], a large effort to develop HIV-1 vaccine has switched to the induction of $CD8^+$ cytotoxic T lymphocyte (CTL) responses against the conserved internal viral antigens, such as Gag and Pol [3,4,5]. Interestingly, the addition of the Env to Gag-Pol in vaccines dramatically increased the capacity of vaccinee to contain SHIV replication [6]. This was not resulted from the different magnitude of T-cell immune responses because both Gag-Pol-Env and Gag-Pol immunized groups mounted comparable T-cell immune responses against Gag and Pol; rather, the induction of additional T-cell immune responses against Env at least partially accounted for the improvement of viral control. Furthermore, the inclusion of Env accelerated the induction of neutralizing antibodies against the challenge virus by 2~3 weeks, which may also have significantly impacted on the viral replication in the Gag-Pol-Env immunized group [6]. In addition, since the neutralizing antibodies could protect target cells from HIV-1 infection, the vaccine effort to induce neutralizing antibodies continues. Env is the only immunogen able to induce neutralization antibodies, it is rationalized that Env is included in the vaccine design [7–14].

Previous study showed that the full-length gp160 of Env was cytotoxic for expressing cells and not suitable for the use as vaccine immunogen. Therefore, the concept to truncate gp160 to gp145 or gp140, which will remove the cytotoxic activity [15] while retaining its ability to induce appreciable antibody immune responses, has been developed. However, it remains unknown how to optimize Env immunogen in vaccine design and what is the modality of vaccination for best use of Env immunogen.

Here we employed a modality of DNA priming/recombinant Tiantan vaccinia boosting to systemically compare the immunogenicity of HIV-1 gp140 and gp145, meanwhile, we also compared modalities of homologous or heterologous priming and boosting. The recombinant Tiantan vaccinia in this study is replicative and derived from the vaccine strain which has been used for decades in billions of Chinese in the campaign to eradicate smallpox. Recently, this replicative vector was used in other vaccine development, such as EBV vaccine [16,17] and its safety profile has been well documented both in the campaign and in the recent clinical trials [16–18].

Materials and Methods

DNA vaccine construction

Humanized gene sequences encoding gp145 of HIV-1_{rl42} (Thailand B) were synthesized as oligonucleotides and then assembled into the whole gene by the overlapping PCR [19]. Gp140 gene was acquired by using PCR amplication from the assembled gp145 gene by exclusion of the transmembrane domain. Both gene sequences were confirmed by sequencing double strands of sense and antisense gene DNAs and then cloned into the expression vector pDRVI-sv1.0 to generate two DNA vaccines, sv140rl and sv145rl. Plasmid vector pDRVI-sv1.0 was generated by inserting a 72-bp SV40-derived enhancer into the upstream of cytomegalovirus immediate-early promoter in pVRC2000 (kindly provided by Dr. Gary Nabel at Vaccine Research Center, NIAID, NIH).

Construction of recombinant Tiantan Vaccinia vectored vaccines

Gp140 and gp145 genes were transferred to pSC65 shuttle plasmid (with a *Lac z* gene as screening marker), which is designed to recombine specifically with *TK* gene of Tiantan vaccinia virus. Subconfluent monolayers of $143TK^-$ cells were grown in Eagle's media

containing 10% fetus bovine serum and 1% Penicillin-Streptomycin-L-glutamine. Then, the cells were washed with Eagle's media containing glutamine and antibiotics in the absence of fetus bovine serum. 5×10^6 pfu vaccinia virus per well were inoculated and incubated for 1 hour at 37°C and 0.5% CO_2. Thereafter, the vaccinia infected cells were further transfected with recombinant shuttle plasmids with Lipofectamine 2000 (CAT#11668-019, Invitrogen). After 48 hour incubation, the transfection media were removed and all wells were covered with 2% melted low melting temperature (LMP) agarose mixed with equal volume 2×Eagle's media containing 100μg/ml x-gal. The blue *Lac Z* positive colonies were picked up and further purified in $143TK^-$ cells under the pressure of selection media (Eagle's media containing 50μg/ml BuDR). The purified recombinant Tiantan vaccinias were confirmed by PCR amplification of inserted gp140 and gp145 and the generated vaccines were designated as rTTV140rl and rTTV145rl, respectively. All rTTVs were expanded in primary chicken embryo cells.

Expression of HIV immunogen by vaccines in vitro

Hela cells were plated in 6-wells plate. 8μg of each plamid of sv140rl or sv145rl, was used to transfect Hela cells. Cells were harvested 48 hours after transfection. Similarly, rTTVs infected primary chicken embryo cells were also harvested 48 hours after infection. All cells were lysed with SDS-PAGE loading buffer and cell lysates were used to determine the expression capacity with Western blotting. 1:100 diluted HIV-1 positive human serum was used as first antibody and 1:1500 diluted HRP-linked goat anti-human IgG(CAT# ZB-2304,Beijing Zhongshan Biotech) was used as second antibody in this assay. Though the Western blotting was carried out in parallel, the transfection and infection were performed separately for different vaccines, thus the cell numbers used for Western blotting may significantly vary. Therefore, this assay was designed to determine whether the immunogens can be properly expressed by vaccines, not the expression efficacy.

Animals and Vaccination

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at China CDC and were performed in accordance with relevant guidelines and regulations. Six-week-old female BALB/c mice were vaccinated as scheduled in Table 1. 100 μg of purified plasmid DNA suspended in 100μl PBS was inoculated intramuscularly into tibialis anterior three times at week 0, 2 and 4. Then at week 6, either 100μg plasmid DNA or a recombinant Tiantan vaccinia virus was injected intramuscularly into tibialis anterior for boosting. The mice were sacrificed 3 weeks after the last inoculation. Sera were collected and stored at 4°C for future quantification of antibodies, and splenocytes were freshly collected for Elispot assay.

Peptide pools

A set of peptides spanning the gp145 gene of consensus B clade (kindly provided by NIH Research and Reference Reagent Program, NIAIDS, NIH) were used as stimuli, the homogenicity between Env of HIV-1_{rl42} and of consensus B is 90%. Peptides (15 mers with overlapping by 11 amino acids with its next peptide) were used at a final concentration of 4μg/ml for each peptide. All peptides were synthesized as free acids and were more than 80% purity. Lyophilized peptides were reconstituted to 1mg/mL in 90% RPMI 1640 with 10% DMSO for the peptide mixture. 4 peptide pools were formulated, including Env 1 (the first 48 peptides), Env 2 (the second 48 peptides), Env 3 (the third 48 peptides), Env 4 (the forth 48 peptides), and used in Elispot assay.

Elispot assay

3 weeks after the final inoculation of DNA vaccine or rTTV vaccines, mice were sacrificed and splenocytes were harvested. IFN-γ Elispot kits (Cat# CT317-T20, U-Cytech Biosciences, the Netherlands) was used to determine vaccine-elicited IFN-γ responses in Balb/c mice. 96-well plates were coated with purified anti-mouse IFN-γ monoclonal antibodies at the concentration of 5μg/ml in 100μl/well, and incubated at 4□ overnight, then washed 4 times with 200μl/well PBST (PBS containing 0.05% Tween20), and blocked with 200μl/well block solution (supplied in the kit). Mice splenocytes were isolated and red blood cells (RBC) were lysed by RBC Lysis Buffer (139.6 mmol/L NH4Cl,16.96 mmol/LTris, pH 7.2). Cells were then washed 2 times with R10 (RPMI-1640 containing 10% fetus bovine serum and 1% Penicillin-Streptomycin-L-glutamine) and re-suspended in R10 complete culture medium. After counting, splenocytes were then adjusted to the concentration of 4×10^6 cells/ml and plated into pre-coated 96-well Elispot plate at 50 μl/well (2×10^5 cells per well) with addition of 50μl peptide pools, the final concentration for each peptide is 4μg/ml (the final concentration of DMSO is 0.04%). 96-well Elispot plates were incubated for 30 hours at 37□ and 5% CO_2. After incubation, the Elispot plates were developed according to the kit instruction. Finally, plates were air-dried and the resulting spots were counted with Immunospot Reader (CTL Inc., Cleveland, OH). Peptide pool specific IFN-γ Elispot responses were considered as positive only when the responses were 4-fold above negative control with no peptide stimulation. Spot forming cells from 4 Env peptide pools were compiled together as the total Elispot result for each mouse.

Quantification of HIV-1_{rl42} envelope-specific binding antibody titers

96-well flat-bottom plates (Costar, NY) were coated at 4°C overnight with 100μl HIV-1_{rl42} gp120 (more than 80% purity, expressed in E.coli and resolved in sodium bicarbonate buffer (pH9.6) at a final concentration of 4μg/ml) and then washed twice with PBS, and blocked at 37°C for 1 hour with block solution (PBS containing 5% skimmed dry milk). Mice sera were serially 2-fold diluted in block solution, and 100μl diluted sera were added to each well. After incubating the plates at 37°C for 1 hour, the plates were then washed 5 times with PBS-T (PBS containing 0.05% Tween20), and 100 μl/well of peroxidase-conjugated anti-mouse immunoglobulin G (diluted 1:2000 in block solution) was added and incubated for 1 hour at 37°C. The wells were washed 5 times with PBS-T again, and 100 μl of OPD (Cat# P9187, Sigma Aldrich) substrate was added and incubated for approximately 10 minutes at room temperature. Color development was terminated by the addition of 50μl/well of 2 N sulfuric acid. Antibody reactivity was then determined by measuring the optical density (OD) at 492 nm with an automated plate reader (Multiscan Ascent, Thermo corporation, Finland). Endpoint titers were determined by the last dilution whose OD was >2 folds than that at the corresponding dilution of mice sera immunized with sv1.0/wild type TTV.

Linear antibody epitope mapping

The method of PLL-ELISA was employed in this study [20,21]. PLL (poly-L-leucine, 30–70 kDa, Sigma Aldrich) was dissolved at the concentration of 40 μg/ml in the sodium bicarbonate buffer, 50μl/well PLL solution was added to 96-well ELISA plate and incubated at room temperature for 1 hour. The plate was washed once with PBS, incubated with 50μl per well of 1% (v/v) glutaraldehyde (Sigma Aldrich) in PBS at room temperature for 15 minutes for activation of PLL and washed twice with PBS. Then 50 μl/well of testing peptides at the concentration of 10μg/ml in PBS was added to the wells pre-coated with PLL and activated with glutaraldehyde. The plate was sealed, incubated overnight at 4°C and then washed twice with PBS. Reactive aldehyde sites were blocked by the addition of 1 M glycine, 200μl/well, for 1 hour at room temperature. The plates were washed twice again with PBS and blocked with 1M glycine,100μl/well, for 1 hour at room temperature, then incubated with 100μl/well

of 0.5% nonfat dry milk:0.5% gelatin (dissolved in PBS) for 1 hour at room temperature. Due to the limited volume of mice sera from each mouse, mice sera from the same group were combined together and diluted at the ratio of 1:100 in PBS containing 5% nonfat dry milk, 50μl/well diluted sera was added into plate in duplicates and incubated at 37°C for 1 hour. The plates were then washed 4 times with PBS-T, incubated with 50μl/well diluted HRP-linked second antibody (1:2000 diluted in PBS containing 2% nonfat dry milk, CAT# ZB-2305, Beijing Zhongshan Biotech) at 37°C for 1 hour, washed 5 times again with PBS-T, and finally incubated with 50μl/well OPD(Sigma Aldrich) substrate at room temperature in dark place for about 10 minutes. The reaction was terminated with 50μl/well 2N sulfuric acid, and the optical density (OD) for each well was read with an automated plate reader (Multiscan Ascent, Thermo corporation, Finland) at 492 nm. Results of experimental settings were displayed as original OD values and >2-fold OD values of mock vector control groups against the corresponding peptide were considered as positive.

Results

Construction and *in vitro* expression of DNA and rTTV vaccines

To compare the immunogenicity between HIV-1_{rl42} gp140 and gp145, we constructed 2 DNA vaccines, sv140rl and sv145rl, and 2 rTTV vaccines, rTTV140rl and rTTV145rl. All the constructs were confirmed by PCR (data not shown). *In vitro* expression of these vaccines were examined by Western blot (Fig. 1). As expected, all these vaccines expressed a protein banded between 130kDa and 170kDa whereas control plasmid or rTTV did not, indicating that inserted *env* genes are able to express corresponding Env. Since this assay was not designed to compare the expression efficacies of different vaccines, the transfection and infection were carried out separately for different vaccines and varied cell number may have been used to prepare cell lysate for Western blotting, thus it is expected we may observe difference of expression between gp140 and gp145 in Fig. 1.

Env-specific T cell immune responses

To determine the immunogenicity, we next immunized mice with these vaccines as scheduled in Table 1. HIV-1_{rl42} Env-specific T cell responses were quantified with IFN-γ based ELISPOT assay after stimulation of splenocytes with HIV-1 consensus B gp145 peptide pools. All the results were subtracted by the background which is resulted from the stimulation with no peptide (<10 SFCs per million cells, supplementary figure 1). For each mouse, Env-specific T cell responses against all 4 peptide pools were compiled together as the total T-cell responses for each mouse and graphed into groups, and the significance of difference among different groups were calculated by using a statistical method of one way analysis of variance (Sigmastat3.1). Results demonstrated that all 4 groups boosted with rTTV (1554±678 SFCs/10^6 splenocytes for the group immunized with sv140rl plus rTTV140rl, 1678±579 SFCs/10^6 splenocytes for sv140rl plus rTTV145rl vaccination, 2053±786 SFCs/10^6 splenocytes for sv145rl plus rTTV140rl vaccination and 2068±635 SFCs/10^6 splenocytes for sv145rl plus rTTV145rl vaccination) based vaccine mounted higher Env-specific T-cell immune responses than that inoculated with DNA vaccine alone (433±112 SFCs/10^6 splenocytes for sv140rl vaccination and 1324±603 SFCs/10^6 splenocytes for sv145rl vaccination) (Fig. 2). Furthermore, no matter DNA vaccine immunization alone or DNA priming/rTTV boosting, priming with gp145 consistently tended to elicit more Env-specific T cells than that with gp140. However, statistical significances were only observed between the groups immunized with sv140rl DNA vaccine alone and all other 5 groups. The highest Env-specific T cell responses were reached in the group primed with sv145rl and boosted with rTTV145rl (Fig. 2).

Env-specific binding antibody immune responses

We next examined binding antibody immune responses. HIV-1_{rl42} gp140- and gp145-specific binding antibodies were quantified by ELISA with bacterial expressed gp120 as the coating antigen. It was observed that the titers of Env-specific binding antibodies elicited by DNA vaccine sv140rl (ranged from 3200 to 12800 with a median of 6400) were higher than DNA vaccine sv145rl (ranged from 1200 to 6400 with a median of 3200). All the groups boosted with rTTVs (titers ranged from 600 to 38400 with a median of 19200 for group sv140rl plus rTTV140rl, 2400–38400 with a median of 9600 for group sv140rl plus rTTV 145rl, 400–19200 with a median of 8000 for group sv145rl plus rTTV140rl and 800–38400 with a median of 19200 for group sv145rl plus rTTV145rl) showed higher binding antibody titers than the groups inoculated with DNA vaccine alone. However, overall there were no statistical significant differences (using the method of one way analysis of variance) among the titers of 6 groups (Fig. 3).

Linear antibody epitope mapping

To further characterize the antibody responses, we performed assay to precisely map the linear antibody epitopes. OD values were used as the measurement of antibody responses against all linear peptides. Data showed that no significant humoral responses were detected in sera from vector control groups (sv1.0 alone or sv1.0 priming/wild type Tiantan vaccinia virus boosting) and the mean OD value of control groups was 0.2 (supplementary figure 2). Interestingly, we observed distinct responding patterns among different groups. Linear peptide epitopes with vigorous humoral responses were identified and clustered in several "*hot regions*" across the full amino acid sequence of HIV-1_{rl42} envelope, including p22~p27 of C1, p76/p77 of V3, p86/p87 and p91~94 of C3, p115~p119 and p121/p122 of C5 and p145~p147 between the 2 HR (heptad repeat) regions (Fig. 4). The intensities of responses against those regions varied among different groups, but, generally, responses in groups boosted with rTTVs mounted higher than that inoculated with DNA vaccine alone. Interestingly, sera raised by gp145 priming but not by gp140 were vigorously reactive to peptide p77, p86/p87, p91/p92/p93/p94 and p115/p116 whereas p76, p121/p122 were more reactive to sera raised by gp140. However, DNA inoculation alone may display different immunogenicity from that in the modality of priming and boosting. For example, gp145 DNA vaccine alone was incapable of whereas gp145 priming plus gp145 boosting are capable of raising vigorous responses to p77, p91/92 and to p117/p119, which implicated that rTTV may present different predominant epitopes from what presented by DNA vaccine. In addition, the order of gp140 and gp145 in the sequential inoculation may exert significant influences on the magnitude of peptide-specific response. Although gp140 plus gp145 and gp145 plus gp140 stimulated similar level of responses to peptide p22-p27, p76, p87, p118 and p145/p146, it was gp145 plus gp140 but not gp140plus gp145 stimulated significantly responses to peptide p77 and p86.

Discussion

Envelope glycoprotein is the primary target for HIV vaccine design. Although the diversity of Env protein poses a grand challenge for generating broad reactive neutralizing antibody responses and T-lymphocyte responses [22,23], Env remains critical in the induction of suppressive immunity in current AIDS vaccine candidates tested in non-human primates [24, 25], and is included in the majority of HIV vaccines, especially in polyvalent vaccines and gp120 based protein vaccines [14].

However, clinical studies demonstrated that antibodies elicited by soluble gp120 have a narrow neutralization specificities, are unable to neutralize primary isolates [26–30] and thereby qualitatively different from those induced by HIV-1 infection [31,32]. A critical deficiency of the gp120 vaccines is the absence of epitopes in the relative conservative protein of gp41

[33,34], Therefore, it is rationalized that gp160 might be a better immunogen than gp120. However, previous study had shown that gp160 is cytotoxic and truncation from its C-terminal to less than 145 kDa could remove its cytotoxicity [15]. Thus, gp140 was thought to be a better vaccine immunogen, as it contains the entire ectodomain of HIV-1 Env and can be secreted from expressing cells. Several vaccine strategies attempted to elicit cross-reactive antibodies by incorporating an oligomeric/trimeric form of gp140 [35–40]. Immunogenicity comparison between gp120 and gp140 showed that sera raised by gp140 exhibited improved cross-reactivity to heterologous Env proteins and greater neutralizing activities compared to what to be raised by gp120 [41].

It remains unknown whether the immunogenicity of gp140 could be further enhanced by including more amino acids at its C-terminal. This study was designed to comprehensively compare the immunogenicity between a gp140 and a gp145 vaccine which added 30 aa at the C-terminal of gp140. Two DNA vaccines and two recombinant Tiantan vaccinia viruses were constructed for this study and the comparisons were performed with a DNA vaccination alone or a Prime-Boost regimen. A number of previous studies have focused on the ability of Env to elicit cross-reactive neutralizing antibodies [15,41–49], however, several primate experiments reminded us to pay attention to specific binding antibodies, which might also play important roles in vaccine induced protection [20,21], and clinical data also identified HIV-specific antibodies but not T cell responses associated with protection [50]. In this study, we first compared the titers of gp120-specific binding antibodies between different groups. Although no significant difference were reached, the mean titer of mice immunized with gp140-encoding DNA vaccine was higher than that in mice vaccinated with gp145-encoding DNA vaccine, indicating that gp140 may be more efficient in elicting gp120-specific binding antibody responses than gp145 when used as DNA vaccine. However, since the gp120 protein used in the ELISA was expressed in bacteria and did not subject to be properly folded, this assay may underestimate the differences among different groups, which may explain why our results are different from what was observed in previous reports [47,49].

In contrast to the responses of binding antibodies, the results of linear antibody epitope mapping showed distinct response patterns among the 6 groups (Fig. 4). Groups immunized with DNA +rTTV showed broader and more vigorous reactivity than groups immunized with DNA only, and it seemed that gp145 could raise antibodies recognizing more linear antibody epitopes and thereby mounted broader antibody responses than gp140. The difference was not due to gp145 has more peptide epitopes at the C-terminal than gp140 because those epitopes primed by gp145 were located within gp140. A more reasonable explanation might be that the different cell membrane anchoring ability of gp140 and gp145 account for this differences, which has been reported before [15]. Consensus B Env derived peptides were used in our assay, which is 90% homogeneous to the Env of HIV-1_{rl42}. The epitopes with specific antibody binding activity in our experiments were mainly located at the high homolog regions: 13 peptides (p23~27/p90~93/p117~119/p121) have 100% homolog in amino acid sequence with HIV-1_{rl42}; 6 peptides(p22/p86~87/p94/p116/p122) have one amino acid different from HIV-1_{rl42} and 3 peptides(p76~p77/p115) have more than two amino acids different from HIV-1_{rl42}(information detailed in supplementary Fig. 3). Therefore, we may underestimate the breadth of humoral responses for HIV-1_{rl42} gp140 and gp145 vaccines at those regions with low homolog. However, this underestimation was not likely to be important for an effective vaccine because the non-cross-reactive epitopes will not be epitopes providing any significant protection for vaccinee.

Two observations should be noticed in our studies. Firstly, DNA vaccination alone may be unable to fully evaluate the immunogenicity of vaccines because we observed that DNA inoculation alone was unable to stimulate vigorous humoral responses to certain epitopes whereas the modality of priming and boosting was capable, thus a strong boost is necessary to

overcome this deficiency. Secondly, we tested the modality of heterologous priming and boosting, we observed that the order of gp140 and gp145 in the sequential inoculation could exert significant influences on the magnitude and specificities of humoral responses. In our experiments, we observed that priming with gp145 and boosting with gp140 raised broader epitope-specific humoral immune responses than that by gp140 priming and gp145 boosting. Therefore, the modality of priming and boosting with heterologous immunogens and the order of inoculations may be highly relevant to the development of HIV vaccine [51]. However, it remains to be addressed the exact underlying mechanism for these observations and how these differences could influence neutralizing activities. In vitro neutralizing assay before and after the absorption of sera by peptides will be able to answer the latter question. Interestingly, based on the crystal structure of gp120 [52], linear peptide epitopes with vigorous humoral responses could be located at both the inner domain (p22~p27/p115~p119/p121/p122) and the outer domain(p76/p77/p86/p87/p91~94) of gp120, which indicates that not only the “outer” epitopes, but also the “inner” epitopes can elicit specific binding antibody response. Besides, the peptide 76 and 77 are located on the tip of V3 loop which partially overlaps with the neutralization epitope identified by the antibody 447-52D [53].

As known, Env-specific T-cell responses also play important role in suppressing HIV replication. One recent study indicated that T cell responses elicted by envelope contributed to vaccine induced protection from challenging viruses carrying heterologous envelope in rhesus monkeys [24]. Clinical data of our lab also showed that HIV-specific CD8 T cell responses targeting envelope are quite high in individuals with chronic HIV-I B’ infection (unpublished data). These results imply that Env-elicited T cell responses may play a role in fighting against HIV-1. In present study, HIV-specific T cell responses induced by gp140 and gp145 were also compared. Results showed that HIV-specific T cell responses of mice immunized with gp145 raised higher T cell responses than that with gp140, significant difference were only observed in the immunization with DNA vaccine alone. This observation is different from previous report in which gp140 elicited a stronger cellular responses than gp145 did [15]. The discrepancy may be explained by the fact that a functional cytotoxicity assay was employed in the previous report, which mainly define the cytotoxic T cells, whereas INF-gamma based Elispot assay was used in our study, which quantified IFN-gamma producing T cells. As known, IFN-gamma production and cytotoxic activity could be two independent events for $CD8^+$ T cells [54,55]. Besides, our data also showed animals primed by sv145rl always had stronger specific T cell responses than those primed by sv140rl (Fig. 2), no matter boosted by rTTV140rl or rTTV145rl, which indicates that the ultimate T cell response of prime-boost regimen may mainly depend on priming stage.

Two limitations should be noticed in this study. First, it remains to be determined whether our data are relevant to the neutralizing activities; Second, during antibody epitope mapping, OD values were used to display the magnitude of antibody responses and may underestimate the differences of antibody responses among different groups. Further studies to address these issues are required.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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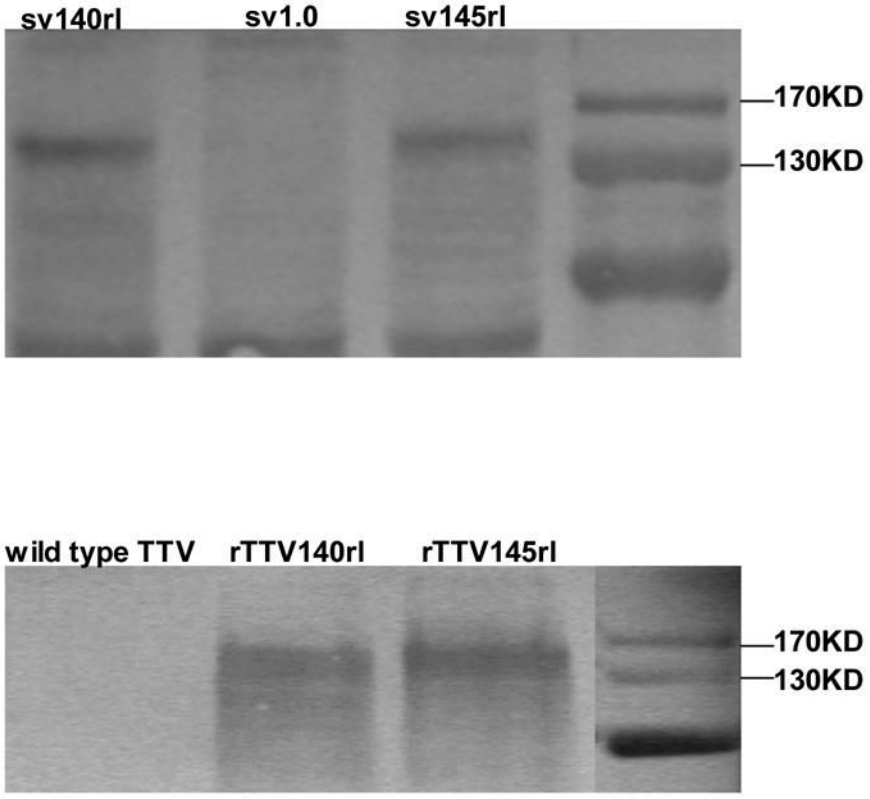


Figure 1.

In vitro expressions of DNA vaccines encoding HIV-1_{rl42} gp140 and gp145 in Hela cells (upper panel) and of rTTVs in primary chicken embryo cells (bottom panel) were assayed by Western Blotting of cell lysate. Blank vector pSV1.0 and wild type Tiantan vaccinia virus was used as mock controls. Transfection and infection were carried out separately for different vaccines, cell numbers used for preparation of cell lysate were varied.

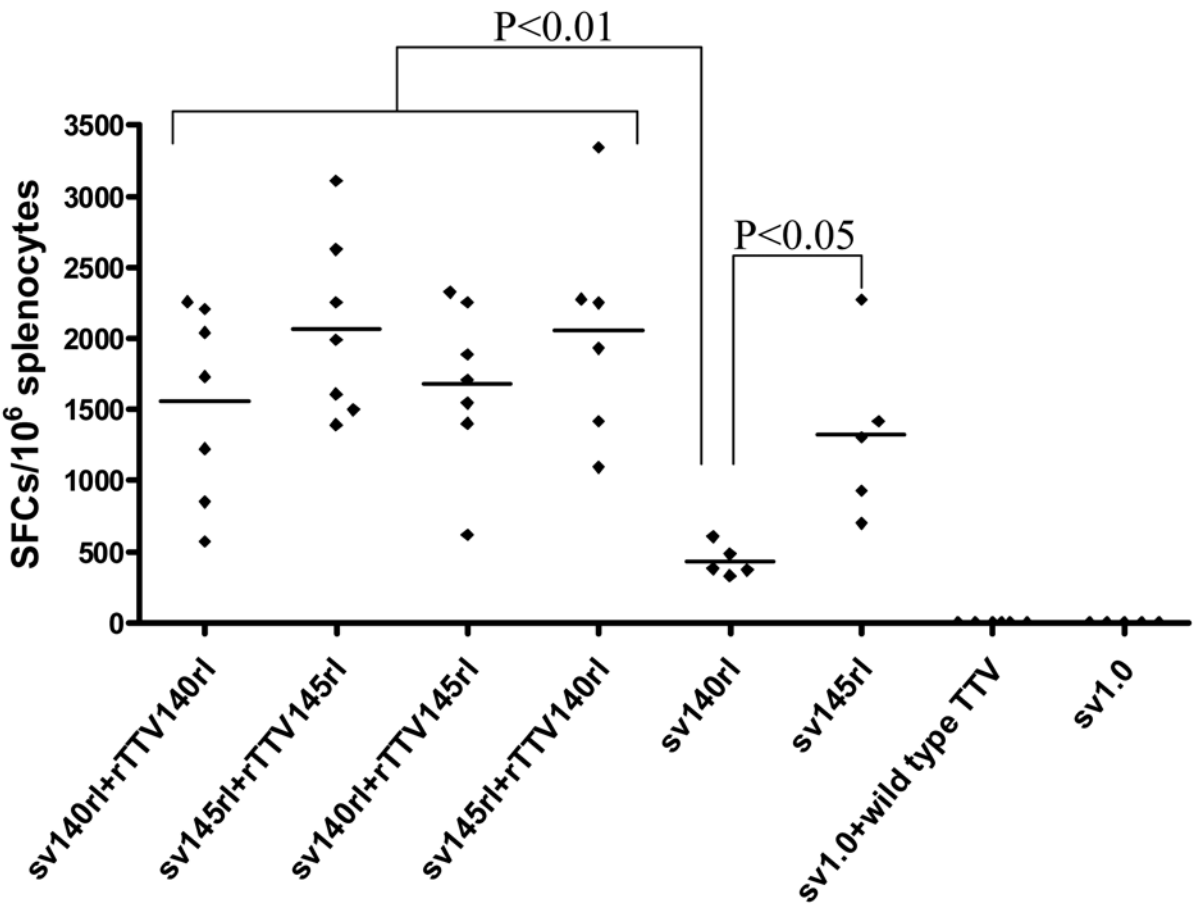


Figure 2.

Env-specific T cell immune responses among different groups. Mice immunized with different regimens of vaccines (as scheduled in table 1) were sacrificed and splenocytes were harvested. Four peptide pools derived from consensus B clade Env were used as stimuli in Elispot assay, T-cell responses for each mouse were compiled from 4 peptide pool stimulations and then grouped in the figure. Positive Elispot control (stimulated with PMA and Inomycin) and negative control (stimulated with no peptides) were also included. Peptide pool specific IFN-γ Elispot responses were considered as positive only when the responses were 4-fold above negative control with no peptide stimulation. Statistical significances were only observed between gp140 and other 5 groups. Control groups immunized with mock vectors raise no T cell immune responses.

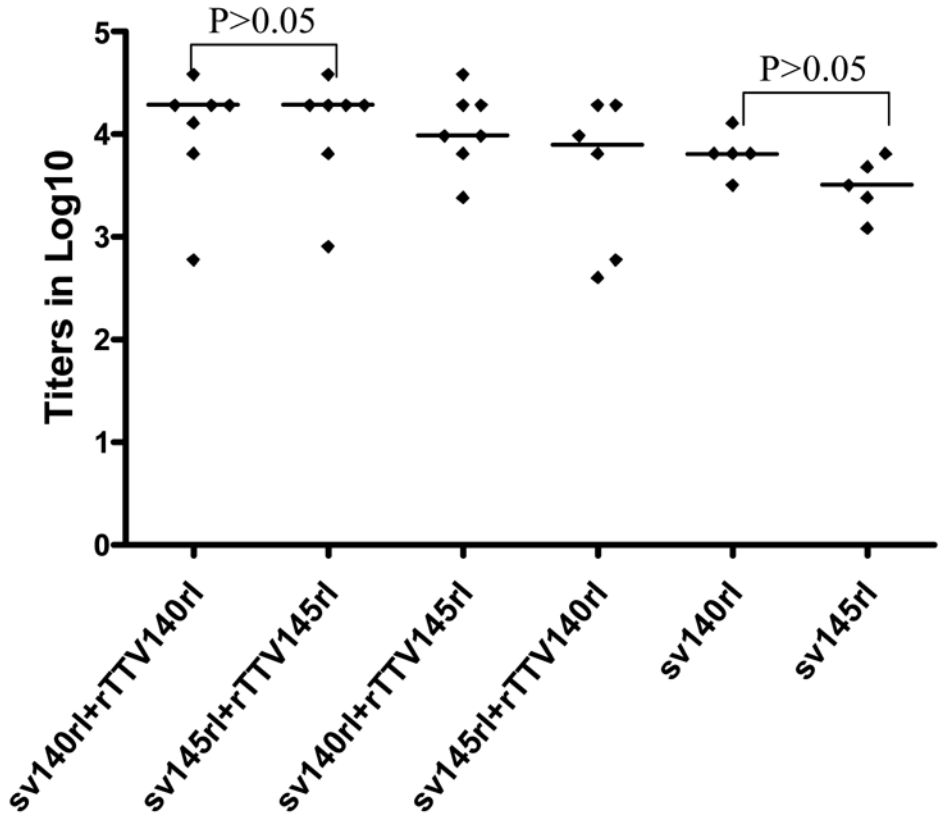
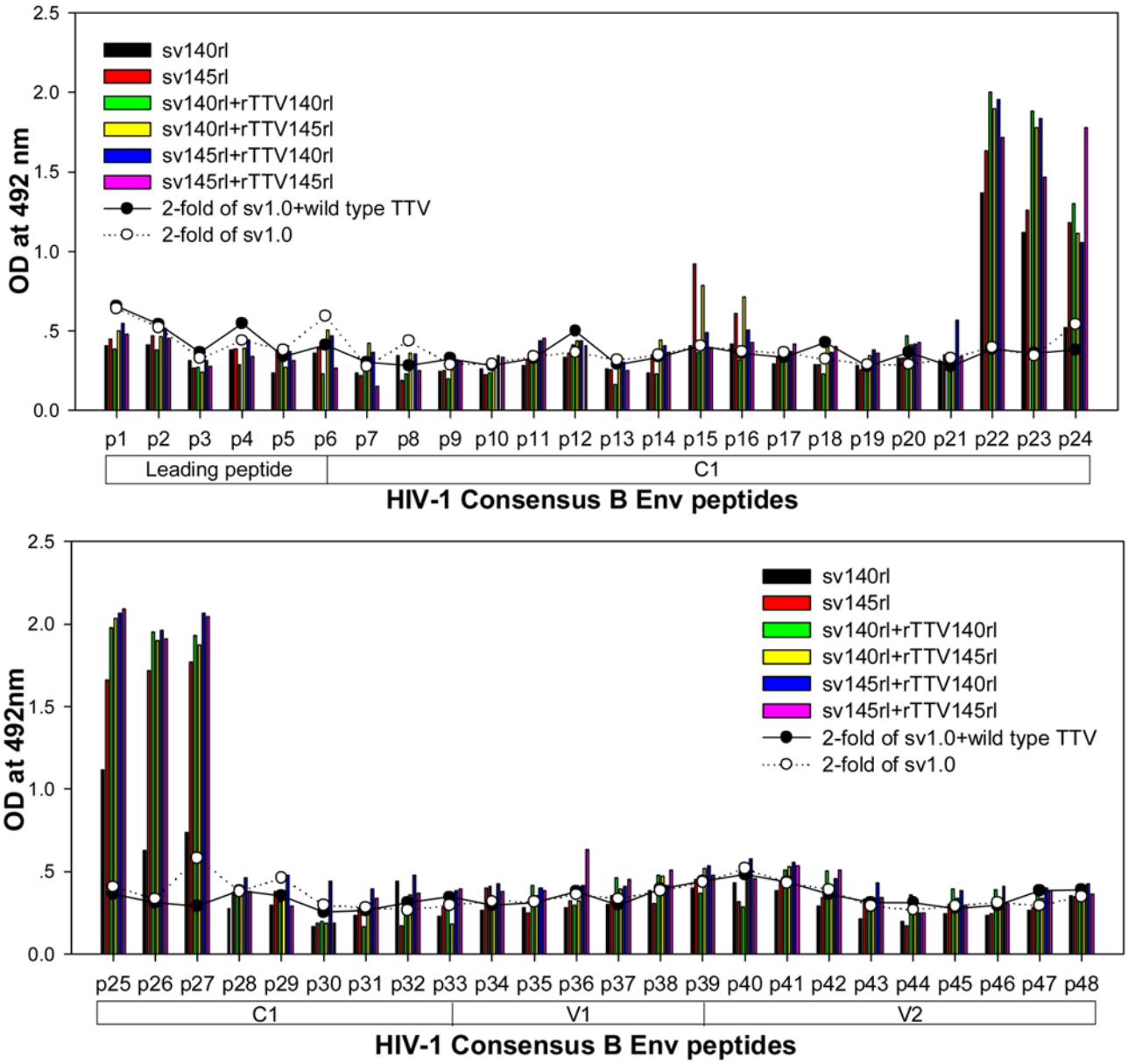
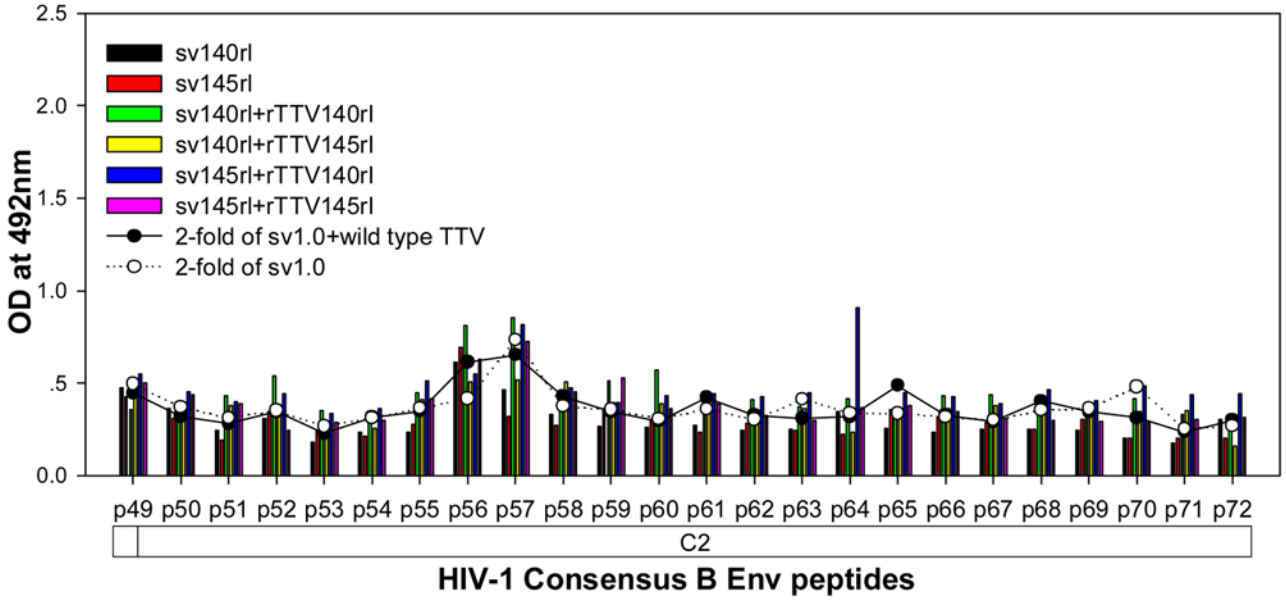
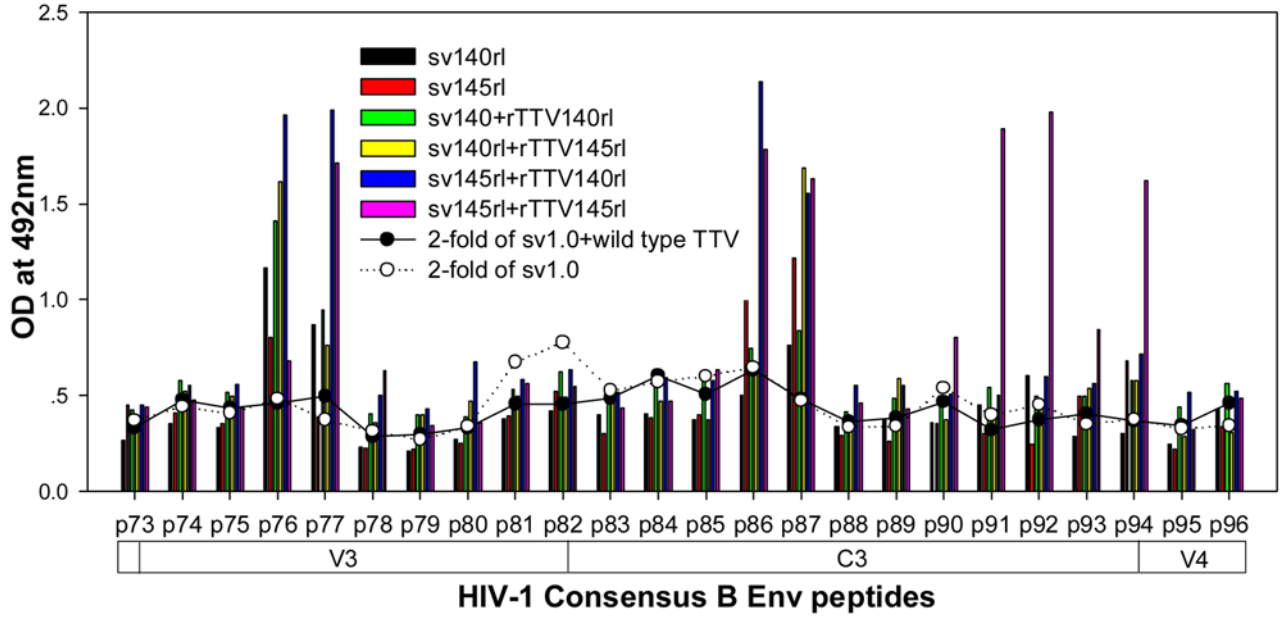


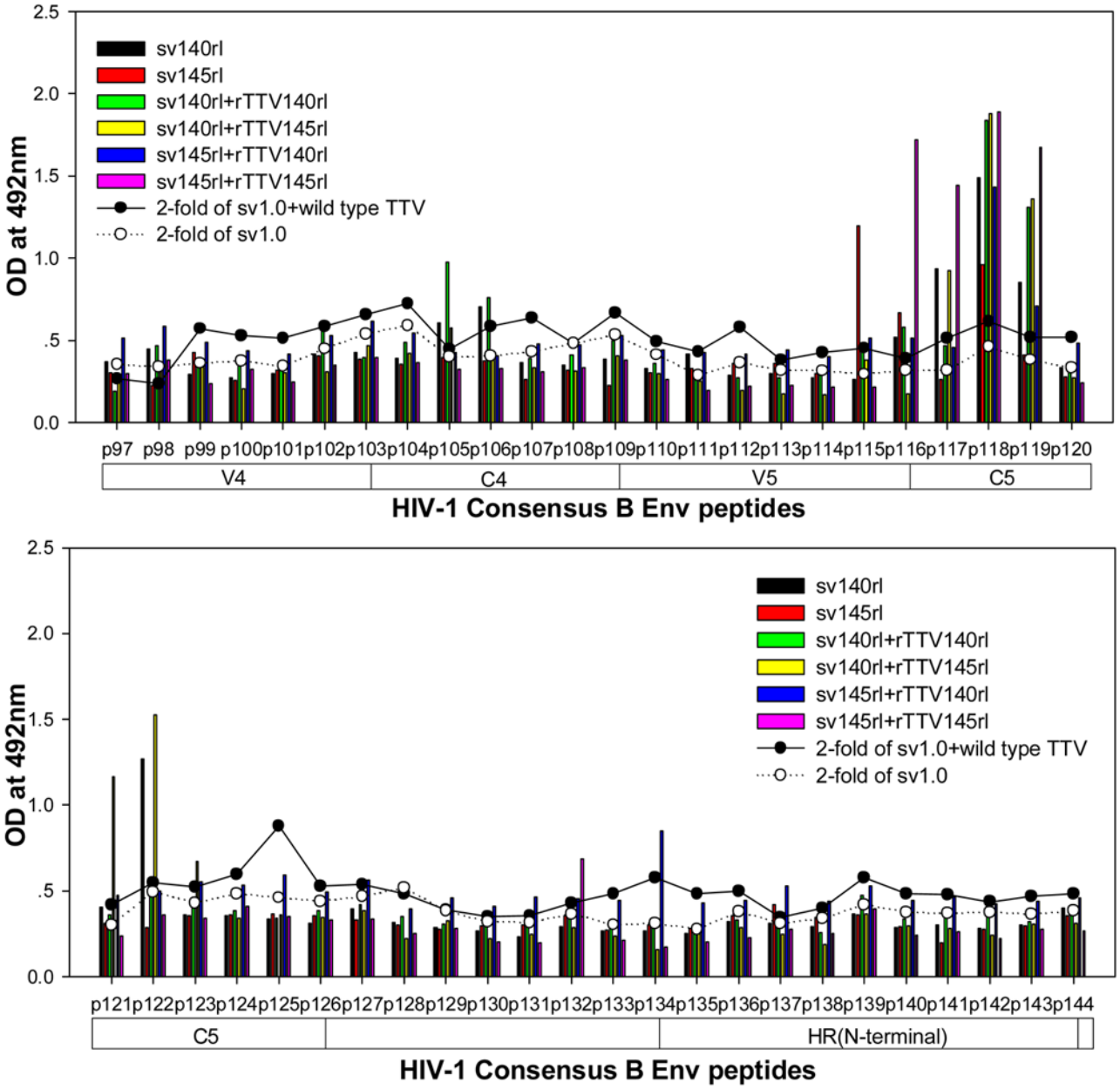
Figure 3.

Gp120-specific binding antibodies among different groups. Mice immunized with different regimens of vaccines (as scheduled in table 1) were sacrificed and sera were collected. Gp120 expressed in *E.coli* was used in ELISA assay and endpoint titers were determined by the last dilution whose OD was >2 folds than that at the corresponding dilution of mice sera immunized with mock control group (sv1.0/wild type TTV). Control groups immunized with mock vectors raise no humoral responses. No significant differences were observed among all 6 groups.









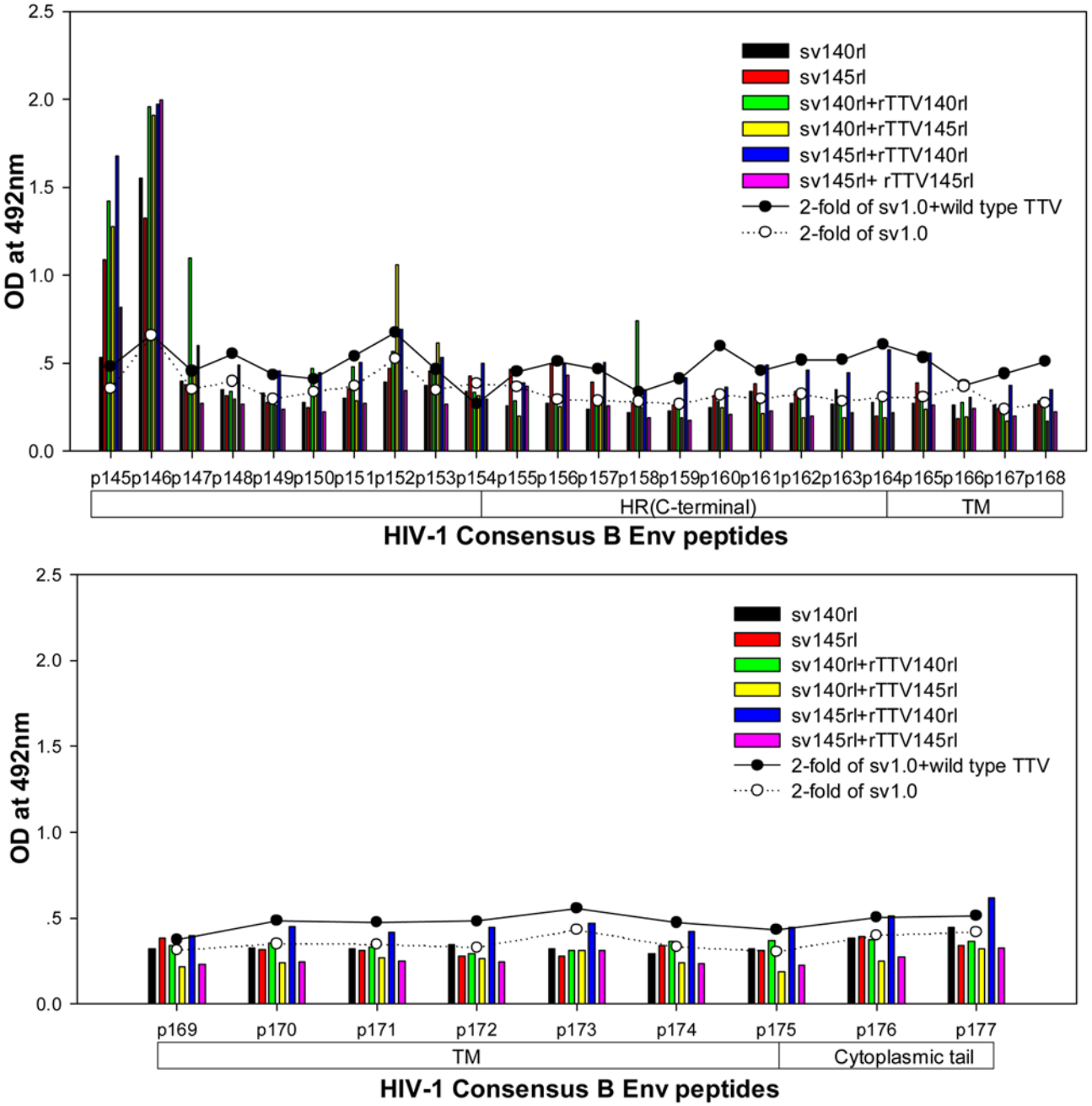


Figure 4.

Results of linear antibody epitope mapping. For each group, the sera from mice in the same group were combined together in equal volume and mixed. The mixed serum was then assayed in duplicated wells. The averages of duplicated original OD values from vaccination groups were graphed into figures. The straight-scatter lines in the figures indicate the threshold value, which is 2-fold of the OD values from mock vector controls against each corresponding peptide (*2-fold of sv1.0* is the cutoff value for groups immunized with DNA only; *2-fold of sv1.0+wild type TTV* is the cutoff value for groups immunized DNA plus rTTV). Though vaccination stimulated no significant responses to the majority of peptides, several highly reactive epitopes were identified across the full amino acid sequences of HIV-1_{rl42} gp145, including p22~p27

of C1, P76/p77 of V3, p86/p87 of C3, p116~p119 of C5 and p145/146 between the 2 HR (heptad repeat) regions.

Table 1

Female Balb/c mice vaccination schedule

		Vaccination and sacrifice schedule				
Group	**No. of Mice**	**Week 0**	**Week 2**	**Week 4**	**Week 6**	**Week 9**
		DNA vaccination (100μg/mouse)				
A	**5**	sv140rl	sv140rl	Sv140rl	sv140rl	sacrificed
B	**5**	sv145rl	sv145rl	Sv145rl	sv145rl	sacrificed
C	**5**	sv1.0	sv1.0	sv1.0	sv1.0	sacrificed
		DNA priming (100μg/mouse)			**Vaccinia boosting** (4×10^6pfu/mouse)	
D	**6**	sv1.0	sv1.0	sv1.0	Wild type TTV	sacrificed
E	**7**	sv140rl	sv140rl	Sv140rl	rTTV140rl	sacrificed
F	**7**	sv140rl	sv140rl	Sv140rl	rTTV145rl	sacrificed
G	**6**	sv145rl	sv145rl	Sv145rl	rTTV140rl	sacrificed
H	**7**	sv145rl	sv145rl	Sv145rl	rTTV145rl	sacrificed