In vivo electroporation in DNA-VLP prime-boost preferentially enhances HIV-1
envelope-specific IgG2a, neutralizing antibody and CD8 T cell responses

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17 **Abbreviations**

18 EP: electroporation

19 DDVV: priming twice with DNA plasmids and boosting twice with HIV-1 VLP

20 VLP: virus-like particles

21 RB: recombinant baculovirus

22 c-di-GMP: cyclic diguanosine monophosphate

23 TCA: trichloroacetic acid

24 BSA: bovine serum albumin

25 SHIV: simian-human immunodeficiency virus

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ABSTRACT

Although *in vivo* electroporation (EP) has been utilized to improve immunogenicity in DNA vaccines alone or in prime-boost regimens with both proteins and viral-vectors, no studies on *in vivo* EP in DNA-VLP prime-boost regimens against HIV-1 have been reported. Previously we developed stably transfected *Drosophila* S2 clones to produce HIV-1 virus-like particles (VLP) and demonstrated that priming mice twice with DNA plasmids encoding HIV-1 gp120 and gag and boosting twice with HIV-1 VLP (i.e. DDVV immunization) elicited both envelope-specific antibody and envelope and gag-specific CD8 T cell responses. However, the potency and the breadth of immunogenicity still need to be improved. In this study we tested the effect of *in vivo* EP during DNA priming on immunogenicity of this DNA-VLP prime-boost regimen. Here we report that although both DDVV and DDVV + EP elicited gp120-specific ELISA-binding antibody responses, average EC₅₀ values of gp120-specific ELISA-binding total IgG, IgG2a, but not IgG1, antibody responses were significantly higher in DDVV + EP than in DDVV. Moreover, while DDVV elicited neutralizing antibody responses against autologous, but not other five, strains tested, DDVV + EP not only elicited significantly higher anti-autologous neutralizing antibody responses, but also cross-neutralizes four of five other HIV-1 strains tested, including two tier 2 strains. Finally, although CD4 and CD8 T cells from both DDVV and DDVV + EP immunizations secreted IFN- γ , IL-2, TNF- α upon HIV-1 envelope peptide stimulation, average HIV-1 envelope-specific CD8 T cells that secreted IFN- γ , IL-2 and/or TNF- α were significantly higher in DDVV + EP than in DDVV. Thus we conclude that DDVV + EP immunization preferentially increases HIV-1 envelope-specific T_H1 cytokine-mediated IgG2a responses and significantly enhances the potency and the breadth of neutralizing antibody responses including tier 2 viruses. Further study on this heterologous regimen in large animals is warranted.

50 **Keywords.** Heterologous prime-boost vaccination, *in vivo* electroporation, HIV-1, IgG
51 subclasses, neutralizing antibody and T cell responses

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53 **Highlights.**

54 1. DDVV + EP immunization preferentially increases HIV-1 envelope-specific IgG2a responses.

55 2. DDVV + EP immunization significantly enhances nAb responses including tier 2 viruses.

56 3. DDVV + EP immunization significantly enhances HIV-1 envelope-specific CD8 T cell
57 responses.

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Introduction

An effective vaccine against HIV-1 requires both humoral and cellular immune responses [1]. The RV144 trial that combined a pox virus-vectored gp120 prime with a gp120 protein boost demonstrated a 31.2% reduction in HIV-1 acquisition at 42 months following initial vaccination [2]. Subsequent immune correlation studies indicate that broadly neutralizing antibody responses were not found to be a correlate of efficacy; whereas IgG antibody responses to V1/V2 or V3 loop of HIV-1 gp120 correlates with decreased HIV-1 transmission risk in RV144 [3, 4]. Immunization of rhesus macaques with live rhesus cytomegalovirus (CMV) vector encoding SIV gag, pol, rev, nef, tat and envelope provides a powerful stimulation of SIV-specific CD8 T cells that control and eliminate virus in 50% of animals, although it does not prevent initial infection [5, 6]. Subsequent study found that SIV protein-expressing rhesus CMV vectors elicited SIV-specific CD8 T cells that recognize unusual, diverse and highly promiscuous epitopes, including epitopes restricted by MHC class II molecules [7]. Therefore, a vaccine regimen that could elicit both broad HIV-1-specific antibody and CD8 T cell responses are highly desirable [8-10].

In vivo electroporation (EP) has been utilized to improve immune responses to DNA vaccines as candidate HIV-1 vaccines in standalone [11-24] or in prime-boost regimens with both proteins [25-30] and viral-vectored [31-35] vaccines in several animal models and, more recently, in human clinical trials [36, 37]. The results clearly show that co-administration of localized electrical fields by *in vivo* EP significantly increases the immunogenicity of DNA vaccines [38, 39]. For example, in standalone DNA vaccines Liu *et al.* tested a HIV-1 envelope DNA vaccine with or without *in vivo* EP in mice and demonstrated that *in vivo* EP augmented cellular and humoral immune responses and allowed a 10-fold reduction in vaccine dose. Furthermore, they showed that *in vivo* EP also recruited large number of macrophages, DCs, T

and B cells to the site of inoculation [12]. Luckay *et al.* demonstrated that in a non-human primate model a 10 to 40-fold increase in HIV-1-specific cellular immune responses and over 100-fold increase in ELISA-binding antibody responses [15]. Vasan *et al.* tested ADVAX, a multigenic HIV-1 DNA vaccine candidate, with or without *in vivo* EP in healthy human volunteers and demonstrated that *in vivo* EP is safe, tolerable and effective in improving the breadth, potency and durability of cellular immune responses to a DNA vaccine [36]. In heterologous DNA-protein prime-boost regimens both Muthumani *et al.* [25] and Zhang *et al.* [26] demonstrated that in small animal models *in vivo* EP induced significantly higher antibody binding titers that neutralized several tier 1 viruses [25] or BC recombinant envelope-pseudotyped viruses [26]. In heterologous DNA-viral vector prime-boost regimens Winstone *et al.* showed that *in vivo* EP of a mixture of DNA vaccines encoding multiple SIV antigens in conjunction with IL-12, followed by boosting with a recombinant Ad5-based vaccine expressing SIV antigens led to partial protection from a repeat low dose mucosal SIVmac239 challenge with strong virus set point control in the majority of animals [31]. Thus, collectively these studies demonstrated that *in vivo* EP induces a transient state of membrane permeability to facilitate uptake of DNA plasmid in the surrounding interstitial spaces and recruits large number of immune cells to the site of immunization [38, 39]. Surprisingly, although *in vivo* EP has been utilized to DNA vaccines alone or in prime-boost regimens with both proteins and viral-vectored vaccines [25-29, 31-37], to our knowledge no studies on the effect of *in vivo* EP on immunogenicity against HIV-1 elicited by DNA-virus-like particles (VLP) prime-boost regimens have been reported.

Previously we developed a novel system to produce HIV-1 VLP using stably transfected *Drosophila* S2 cell clones [40]. We demonstrated that the amount of HIV-1 VLP produced by S2

cell clones is comparable to that produced by insect cells infected with recombinant baculoviruses (RB) [41] and much higher than those produced by mammalian cells [42-47]. Unlike HIV-1 VLP produced by insect cells infected with RB, culture supernatants produced by stably transfected S2 cell clones are not contaminated with recombinant viruses, which makes downstream purification process simpler. We also demonstrated that priming mice twice with DNA plasmids encoding HIV-1 gp120 and gag and boosting them twice with stable *Drosophila* S2 transfectant-produced VLP elicited both envelope-specific antibody responses and envelope and gag-specific CD8 T cell responses [40]. However, the potency and the breadth of immune responses still need to be improved.

Therefore, in this study we tested the effect of *in vivo* EP during DNA priming on immunogenicity in this heterologous DNA-VLP prime-boost regimen. Post-immune sera and splenocytes were collected after the second boost. HIV-1 envelope-specific ELISA-binding and neutralizing antibody responses as well as envelope-specific CD4 and CD8 T cell responses were carefully evaluated.

Materials and Methods

Cell lines. Stably transfected *Drosophila* S2 cell clone B2 was previously generated in our laboratory and cultured as described before [40]. The 293 FT cell line was bought from Invitrogen Life Technologies and cultured as described previously [48]. TZM.bl cells were obtained from the NIH AIDS Research and Reference Reagent Program (ARRRP; Germantown, MD) [49, 50] and mouse fibroblast cell line L929 (Cat# CCL-1) were purchased from American Tissue Culture Company (ATCC, Manassas, VA) [51]. Both cell lines were cultured in complete DMEM media supplemented with 10% FBS, 1mM sodium pyruvate, 2mM L-glutamine and penicillin/streptomycin [100U/ml each] with CO₂ at 37°C.

Antibodies. Human anti-gp120 antibody 2G12 and mouse anti-HIV-1 Gag p24 antibody 183-H12-5C were obtained from the ARRRP. Human anti-gp120 antibodies VRC01, PGT128 and 10-1074 and control antibody 100F4 (a human anti-H5 hemagglutinin antibody) were produced and purified in our laboratory by using stably transfected S2 clones as described before [52]. llama antibody JM4 [53] was produced and purified in our laboratory by using prokaryotic expression and Ni-NTA affinity chromatography. PreCP-conjugated anti-CD4, allophycocyanin (APC)-conjugated anti-CD8, fluorescein isothiocyanate (FITC)-conjugated anti-IFN- γ , phycoerythrin (PE)-conjugated anti-IL-2, phycoerythrin (PE)-Cy7-conjugated anti-TNF- α , purified anti-CD28 and anti-CD49d antibodies were all bought from BD Bioscience (Mountain View, CA).

Gene constructs. Plasmids CMV/R-gp120 (consensus B) were generated for mammalian expression in our laboratory previously [40].

Production of HIV-1 VLP by a stable S2 clone. HIV-1 VLP production and quantification were as described before [40, 52].

Transfection and western blot analysis. To test the mammalian expression of gp120, L929 cells (10^5) were transiently transfected with 1.6 μ g of plasmid CMV/R-gp120 (consensus B) using Lipofectamine® LTX & PLUS™ Reagent (Invitrogen). Culture supernatants were harvested 2 days post transfection, precipitated with trichloroacetic acid (TCA), and resuspended in PBS. Samples were loaded and separated through 10% SDS–PAGE and then transferred onto PVDF membranes (Millipore). The membranes were blocked in TBST containing nonfat dry milk (5%) and sequentially probed with the antibodies specific for HIV-1 gp120 as well as for β -actin control. Antigens were visualized with HRP-conjugated anti-human IgG antibody (Abcam) or HRP-conjugated anti-mouse IgG antibody (ICL), respectively, and follow by EZ-ECL substrate staining (Thermo).

FACS analysis. To test the antigenicity of consensus B HIV-1 envelope proteins on the surface of a stably transfected S2 clone B2, stably transfected cells were incubated with a panel of human monoclonal antibodies VRC01, 2G12, PGT128 and 10-1074, a llama antibody JM4 or control antibody 100F4 for 1 hour on ice. Then cells were subsequently washed twice with FACS buffer (PBS containing 1% FBS and 0.02% NaN_3) and stained with FITC-conjugated anti-human IgG antibody (Sigma) or mouse anti-his tag antibody followed by PE-conjugated goat anti-mouse IgG antibody (Sigma) for another 30 minutes on ice. Cells were then washed and fixed with 2% formaldehyde in FACS buffer. FACS analysis was performed with a LSRII flow cytometer (BD Biosciences).

Immunization and sampling. Animal protocols were all approved by the Institutional Animal Care and Use Committee of the Institut Pasteur of Shanghai (Approval No. A2011014 & A2014011). Six to eight weeks old female BALB/c mice were purchased and housed as described previously [40]. Mice were divided into three groups randomly (6 or 9 mice per group) for the immunization. DNA immunizations were performed after anesthetization with 65 mg/kg pentobarbital sodium. Mice in group 1 were injected intramuscularly (i.m.) in both hind legs or subcutaneously (s.c.) with 100 µl of PBS. Mice in group 2 were i.m. primed twice with 50 µg of plasmid CMV/R-gp120 (consensus B) and boosted twice subcutaneously (s.c.) with HIV-1 VLP (consensus B) equivalent to 5 µg of HIV-1 gp120 plus 5 µg of c-di-GMP adjuvant [54] . **Mice in group 3 were primed and boosted in the same way as in group 2 except that during the priming DNA were delivered by *in vivo* EP using the EPT-I delivery device (TERESA, Shanghai, China). A central injection needle along with a needle electrode pair with 5 mm spacing in between was inserted into both hind legs shaved. And the DNA vaccination process consisted of 6 pulses at 60V constant voltage, each with 50 ms duration and 1 s interval [55, 56].** DNA prime were given on day 0 and 21 followed by HIV-1 VLP/c-di-GMP boost on day 49 and 77. Seven days before the first prime and ten days after the second boost, serum samples were collected by retro-orbital plexus puncture, heat inactivated and stored in aliquots at -80°C. Spleens were harvested 10 days after the boost and splenocytes were isolated for intracellular cytokine staining (see below).

ELISA. Total IgG, IgG1, and IgG2a antibody responses against consensus B gp120 were measured as described before with HRP-conjugated anti-mouse IgG (1:5,000) (ICL), anti-mouse IgG1 (1:500) or anti-mouse IgG2a (1:500) antibodies (SBA), respectively [40]. EC₅₀ value to a given serum was determined as a serum dilution that results in 50% of the maximum anti-HIV-1 gp120 binding.

HIV-1 pseudotypes and a single-cycle infectivity assay. HIV-1 pseudotypes were generated and single-cycle infectivity assay was accomplished as described previously [40, 48].

Intracellular cytokine staining (ICS). ICS was performed as described before [40], except that splenocytes were stimulated with a mixture of ten 15-mer peptides (2.5 µg of each peptide/ml) derived from consensus B HIV-1 envelope protein (Table 1) [44], along with anti-mouse CD28 and anti-mouse CD49d antibodies. A total of 10^6 cells were acquired per sample on a LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software.

Statistics. Data analyses were performed with GraphPad Prism v5.0. An unpaired Student *t* test was utilized to compare two sets of data. Exact Spearman rank correlation method was used to assess the correlations between the ELISA-binding antibody responses and between neutralizing antibody responses. All *P* values are two-tailed.

Results

Immunogen characterization

To characterize DNA plasmid expressing consensus B gp120, we transfected mouse cell line L929 with or without DNA plasmids encoding consensus B gp120 and/or HIV-1 rev. Fig. 1A shows that HIV-1 gp120 were produced in the mouse cell line and secreted into the culture supernatants with or without co-transfection of gene encoding HIV-1 rev.

Previously, we stained stably transfected *Drosophila* S2 clones using human anti-HIV-1 envelope antibodies 2G12, VRC01, b12 PG16 and 4E10 along with control antibody 100F4 to determined the antigenicity of HIV-1 VLP. We found that except for PG16 epitope, epitopes 2G12, b12, VRC01 and 4E10 tested are present on spikes of HIV-1 VLP produced by S2 clones [40]. Since then, many new broadly neutralizing monoclonal antibodies that recognize different epitopes on HIV-1 envelope have been discovered [57]. So to further characterize the antigenicity of HIV-1 VLP, in this study, we stained stably transfected *Drosophila* S2 cell clone with three new antibodies JM4, 10-1074 and PGT128 along with 100F4 control, VRC01 and 2G12. JM4 is an Llama single domain antibody that recognizes an epitope comprising both CD4 and coreceptor binding sites [53]. 10-1074 and PG128 are two human antibodies that recognize high mannose patch epitope centered on N137, N301, N332 and N397 glycans [58, 59]. Fig. 1B shows that stably transfected S2 cell clone can be well stained by all five anti-envelope antibodies, but not control antibody 100F4. Thus we concluded that the broad neutralization epitopes 2G12, VRC01, JM4, 10-1074 and PG128 are preserved on spikes of HIV-1 VLP produced by S2 cell clones.

EP during DNA priming in the DDVV immunization significantly increases total IgG and preferentially enhances IgG2a ELISA-binding antibody responses against HIV-1 envelope

Fig. 2 summarizes the immunization and sampling schedule as well as assays to measure HIV-1 specific antibody and T cell responses. Fig. 3A shows that although both DDVV and DDVV + EP immunizations elicited consensus B gp120-specific ELISA-binding total IgG antibody responses as compared to PBS control group ($P < 0.001$ for both groups), average EC_{50} was significantly higher in DDVV + EP group ($4,829 \pm 527$) than in DDVV group ($2,575 \pm 268$) ($P = 0.006$). Thus, we concluded that *in vivo* EP during DNA priming significantly enhances HIV-1 gp120-specific total IgG responses elicited by DDVV immunization.

Fig. 3B shows that both DDVV and DDVV + EP sera exhibited consensus B gp120-specific ELISA-binding IgG1 antibody responses as compared to control group ($P = 0.002$ for both groups) and no significant difference in EC_{50} values ($6,336 \pm 1,101$ in DDVV vs. $7,630 \pm 1,216$ in DDVV + EP) between the two groups ($P = 0.4721$). In contrast, average EC_{50} value in consensus B gp120-specific ELISA-binding IgG2a antibody responses was significantly higher ($19,460 \pm 5,945$) in DDVV + EP group than ($1,654 \pm 634$) in DDVV group ($P = 0.032$) (Fig. 3C). Interestingly, there was a positive correlation between EC_{50} values of anti-HIV-1 gp120 total IgG and IgG2a responses in DDVV + EP group (Fig. 4A), but not in DDVV group (Fig. 4B). Thus taken together these results indicate that *in vivo* EP during DNA priming in DDVV immunization preferentially enhances IgG2a responses.

In vivo EP in DNA priming in the DDVV immunization significantly enhances neutralizing antibody responses against diverse HIV-1 strains

To test the effect of *in vivo* EP in DNA priming on neutralizing antibody responses elicited by DDVV immunization, an HIV-1 pseudotype-based neutralization assay was performed against a panel of HIV-1 pseudotypes along with VSV-G pseudotype control. The panel consists of HIV-1 envelopes derived from clade A (Q168), clade B (consensus B, HxBc2 and Yu2), clade

C (consensus C) and CRF01_AE recombinant (CNE3). Among them Q168 and Yu2 belong to tier 2 strains. Fig 5 shows that although DDVV immunization elicited anti-autologous consensus B specific neutralizing antibody responses with average 42.5% neutralization activity at 1:50 serum dilution as compared to PBS control ($P = 0.0002$), it failed to cross-neutralize all other five HIV-1 strains. In contrast, DDVV + EP immunization not only elicited enhanced anti-autologous consensus B specific neutralizing antibody responses with average 59.3% neutralization activity at 1:50 serum dilution as compared to DDVV immunization ($P = 0.0347$), but also cross-neutralizes four other HIV-1 strains HxBc2, Yu2, Q168 and CNE3 with average 42.93, 32.5, 47.98, and 39.31% neutralization activity at 1:50 serum dilution, respectively. Statistically, all are significantly different from DDVV immunization with P values of 0.0186, <0.0001 , <0.0001 and 0.0057, respectively. Both DDVV and DDVV + EP immunizations did not elicit cross-neutralization activity against consensus C pseudotypes and VSV-G pseudotype control. Interestingly, in DDVV + EP group there are positive correlations between neutralization activities against autologous consensus B and Yu2 strains ($R^2 = 0.7119$) (Fig. 6A) and against autologous consensus B and Q168 strains ($R^2 = 0.4447$) (Fig. 6B). Thus we conclude that the *in vivo* EP in DNA priming in the DDVV immunization significantly enhances neutralizing antibody responses against a number of autologous and heterologous envelope proteins including tier 2 strains.

In vivo EP in DNA priming in DDVV immunization significantly enhances HIV-1 envelope-specific CD8, but not CD4, T cell responses

To test the effect of *in vivo* EP in DNA priming on T cell responses elicited by DDVV immunization, splenocytes from DDVV or DDVV + EP immunized and PBS control mice were assayed against a mixture of 10 15-mer peptides derived from consensus B envelope protein

(Table 1) using intracellular cytokine staining for IFN- γ , IL-2 and TNF- α in CD4 or in CD8 (Fig. 7) T cells. Compared to CD4 T cells from PBS control mice, CD4 T cells from DDVV or DDVV + EP immunized mice exhibited higher, but statistically insignificant, HIV-1 envelope peptide-specific responses; and between DDVV and DDVV + EP mice there are also no significant differences (data not shown). Fig. 7A shows the gating strategy of single or double cytokine-secreted CD8 T cells upon peptide stimulation. CD8 T cells from DDVV or DDVV + EP immunized mice exhibited significantly higher HIV-1 envelope peptide-specific responses than PBS control mice. Except for CD8 T cells that secreted IL-2, on average 0.314 and 1.151% of CD8 T cells secreted IFN- γ , 0.113 and 0.211% secreted IL-2, 0.392 and 1.29% secreted TNF- α , 0.035 and 0.177% secreted both IFN- γ and IL-2, 0.231 and 1.00% secreted both IFN- γ and TNF- α , and 0.024 and 0.170% secreted both TNF- α and IL-2 in DDVV or DDVV + EP immunized mice, respectively (Fig. 7B and G). Importantly, except for CD8 T cells that secreted IL-2, between DDVV and DDVV + EP groups there were statistically significant differences ($P = 0.0032$ for IFN- γ ; $P = 0.0027$ for TNF- α ; $P = 0.0127$ for both IFN- γ and IL-2; $P = 0.0024$ for both IFN- γ and TNF- α ; and $P = 0.0091$ for both TNF- α and IL-2) (Fig. 7B and G).

Discussion

Although *in vivo* EP has been utilized to improve immune responses to DNA vaccines alone or in prime-boost regimens with both proteins and viral-vectored vaccines against HIV-1 [11-29, 31-37], to our knowledge no studies on the effect of *in vivo* EP on immunogenicity against HIV-1 elicited by DNA-VLP prime-boost immunizations have been reported. Therefore, in the present study we tested the effect of *in vivo* EP during DNA priming on immunogenicity in the heterologous DNA-VLP prime-boost immunization, in which HIV-1 VLP was produced by a stably transfected Drosophila S2 cell clone as we described before [40]. We primed mice twice with DNA plasmid encoding consensus B gp120 with or without *in vivo* EP and boosted them twice with HIV-1 VLP expressing the full length of consensus B HIV-1 gp160 plus c-di-GMP adjuvant along with mice injected with PBS control.

One interesting finding in this study is that *in vivo* EP during DNA priming not only significantly increases anti-HIV-1 gp120 total IgG responses, but also preferentially enhances anti-HIV-1 gp120 IgG2a, but not IgG1, responses (Fig. 3). IgG subclass switching is known to be strongly influenced by the prevailing cytokine environment and by the nature of the stimulating antigen [60-64]. For example, IL-4, a T_H2 cytokine, preferentially induces switching to IgG1 and IgE [60]; whereas IFN- γ , a T_H1 cytokine, preferentially induces switching to IgG2a, 2b and 3 [64]. Here we showed *in vivo* EP during DNA priming also preferentially induces IgG2a switching. Indeed, we also found that DDVV + EP induces significantly higher percentages of HIV-1 envelope-specific IFN- γ -producing CD8 T cells compared to those induced by DDVV immunization (Fig. 7). Thus, tentatively *in vivo* EP during DNA priming may promote antigen processing and presentation, which results in more HIV-1 envelope-specific CD8 T cells to produce IFN- γ . The latter in turn induces IgG2a switching in HIV-1 envelope-

specific B cells. Furthermore, among IgG subclasses, IgG2a and 2b are generally considered to be the most potent for activating effector responses and dominate antiviral immunity [65, 66]. Indeed several recent studies on anti-HIV-1 or anti-influenza HA broadly neutralizing human/mouse chimeric antibodies clearly demonstrated the requirement of interaction between the IgG2a Fc region and the FcγR for protection against HIV-1 or influenza virus *in vivo* [67-69]. Moreover, in the follow-up immune correlation studies of RV144, human IgG1 (corresponding to mouse IgG2a) and human IgG3 responses to V1/V2 loop of HIV-1 gp120 have been shown to correlate with decreased HIV-1 transmission risk [70, 71]. Thus, the demonstration of *in vivo* EP during DNA priming in heterologous DNA-VLP prime-boost preferentially increases anti-HIV-1 envelope-specific IgG2a responses could have an important implication in vaccine efficacy.

Another interesting finding in this study is that *in vivo* EP during DNA priming not only significantly increases neutralization potency against autologous consensus B HIV-1 strain, but also broadens neutralization breadth against heterologous and even heterosubtypic HIV-1 strains including tier 2 strains, although the neutralization potency still needs to be improved (Fig. 5). Passive infusion with broadly neutralizing monoclonal antibodies has demonstrated efficacy as both preventive and therapeutic agents in humanized mouse and simian-human immunodeficiency virus (SHIV) rhesus macaque models [72-81], more recently in chronic HIV-1 infected individuals [82]. It is conceivable that broadly neutralizing antibody responses, if they are elicited by vaccinations, should play an important role in immune protection. Thus, the demonstration in this study that *in vivo* EP during DNA priming significantly increases neutralization potency and breadth of immune sera elicited by heterologous DNA-VLP vaccination is very encouraging. However, due to limited amount of immune sera in mice in this study we only tested neutralization activity of immune sera against six HIV-1 pseudotypes along

with VSV-G control. Nevertheless, our results warrant more comprehensive testing of neutralization potency and breadth of DDVV + EP sera in the future. Montefiori's group [83] described a global panel of HIV-1 reference strains for standardized assessment of vaccine-induced neutralizing antibodies. Therefore, it is better to test rabbit and monkey immune serum samples against this global panel. Finally, by using competitive inhibition assay Zhang *et al.* showed that neutralization activity against BC recombinants elicited by *in vivo* EP in the DNA-protein prime-boost regimen is mediated by CD4BS antibodies [26]. Thus it is interesting to determine what neutralization epitopes are recognized by DDVV + EP sera.

Still another finding in this study is that *in vivo* EP during DNA priming significantly enhances HIV-1 envelope-specific CD8 T cells that secreted IFN- γ , IL-2 and/or TNF- α (Fig. 7). It has been shown that immunization of rhesus macaques with live rhesus cytomegalovirus vector encoding SIV gag, pol, rev, nef, tat and envelope provides a powerful stimulation of SIV-specific CD8 T cells that control and eliminate virus in 50% of animals [5, 6]. Thus, although as a delivery vehicle replication competent rhesus CMV vector can hardly be compared to *in vivo* EP during DNA priming in this study, the demonstration that DDVV + EP immunization significantly enhances HIV-1 envelope-specific CD8 T cells should still have important implications in vaccine efficacy.

Conclusion

Taken together our results indicate that *in vivo* EP during DNA priming in DDVV immunization preferentially increases HIV-1 envelope-specific T_H1 cytokine-mediated IgG2a responses and significantly enhances the potency and the breadth of neutralizing antibody responses including tier 2 viruses as well as HIV-1 envelope-specific CD8 T cell responses. Thus,

our results warrant further comprehensive studies on this heterologous DDVV + EP regimen in large animals.

Authors' contribution

PZ (Paul Zhou), XH designed study. XH, QQZ, XXH performed experiments. YFS and LFY provided critical reagents. PZ (Paul Zhou), XH, PZ (Ping Zhu) analyzed data. PZ (Paul Zhou) and XH wrote the manuscript with the help of all the authors. All authors read and approved the final manuscript.

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Table.1 The list of a panel of 10 15-mer peptides used for intracellular cytokine staining^a

13-27 ^b (8766 ^c): LWRWGTMLLGMLMIC	113-127 (8791): QSLKPCVKLTPLCVT
305-319 (8839): KSIHIGPGRAFYYTG	353-367 (8851): GNKTIVFNQSSGGDP
412-426 (8866): CRIKQIINMWQEVGK	459-473 (8878): TEIFRPGGGDMRDNW
491-505 (8886): APTKAKRRVVQREKR	659-673 (8928): KWASLWNWFDITNWL
775-789 (8975): IVELLGRRGWEVLKY	823-837 (8969): VIEVVQRACRAILHI

^a The 15 mer peptides were derived from consensus B of HIV-1 envelope sequence

^b The positions of amino acid residues of peptides

^c The NIH AIDS Research and Reference Reagent Program catalog numbers of each peptide in parenthesis.

Figure Legends

Fig. 1 Immunogen characterization. A. western blot analysis of consensus B gp120 in culture supernatants. Env: supernatants from L929 cells transfected with DNA plasmid encoding consensus gp120 alone; Env + rev: supernatants from L929 cells transfected with DNA plasmids encoding consensus B gp120 and rev. Anti-gp120 antibodies: VRC01 and 2G12; anti-beta-actin antibody (Sigma). B. FACS analysis of cell surface expression of consensus B gp160 using a panel of anti-gp120 monoclonal antibodies VRC01, JM4, 2G12, 10-1074 and PGT128 along with anti-influenza HA control antibody 100F4.

Fig. 2 Schematic diagram of DDVV and DDVV + EP immunizations and sampling. PBS: PBS control; i.m. intramuscularly; s.c. subcutaneously; EP: *in vivo* electroporation.

Fig. 3 ELISA-binding antibody responses elicited in mice. Anti-HIV-1 envelope gp120-specific total IgG responses (A), IgG1 responses (B), and IgG2a responses (C). EC₅₀: reciprocal serum dilutions that result in 50% of maximum binding.

Fig. 4 Correlative analysis of anti-HIV-1 envelope gp120 responses. Correlation between total IgG and IgG2a responses in DDVV + EP immunization (A) or in DDVV immunization (B).

Fig. 5 Neutralizing antibody responses elicited with DDVV or DDVV + EP immunization. Neutralization activity of immune serum samples at 1:50 dilution against six HIV-1 pseudotypes (consensus B, HxBc2, Yu2, Q168, CNE3, consensus C) and VSV-G pseudotypes control. The percent neutralization was calculated as follows: (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. Statistical analysis was performed between the means $\pm$ the SEM of a given pseudotype.

Fig. 6 Correlative analysis of neutralizing antibody responses. Correlations between autologous strain and tier 2 strain Yu2 (A) or Q168 (B) in DDVV + EP immunization.

Fig. 7 Intracellular cytokine staining analysis of CD8 T cell responses against a panel of HIV-1 envelope peptides. A. Gating strategies of percentages of CD8 T cells that secreted IFN- γ , IL-2 and/or TNF- α . B to D. The percentages of CD8 T cells that secreted IFN- γ , IL-2, TNF- α . E to H. The percentages of CD8 T cells that secreted both IFN- γ and IL-2, IFN- γ and TNF- α , or IL-2 and TNF- α .

Figure 1

Figure 2

Animal groups

- 1- PBS (i.m)
- 2- HIV-1 consensus B gp120 (50µg, i.m.) + consensus B VLP/c-di-GMP (5µg each, s.c.)
- 3- HIV-1 consensus B gp120 (50µg, i.m. + EP) + consensus B VLP/c-di-GMP (5µg each, s.c.)

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7