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UV-inactivated vaccinia virus (VV) in a multi-envelope DNA-VV-protein (DVP) HIV-1 vaccine protects macaques from lethal challenge with heterologous SHIV

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Abstract

The pandemic of HIV-1 has continued for decades, yet there remains no licensed vaccine. Previous research has demonstrated the effectiveness of a multi-envelope, multi-vectored HIV-1 vaccine in a macaque-SHIV model, illustrating a potential means of combating HIV-1. Specifically, recombinant DNA, vaccinia virus (VV) and purified protein (DVP) delivery systems were used to vaccinate animals with dozens of antigenically-distinct HIV-1 envelopes for induction of immune breadth. The vaccinated animals controlled disease following challenge with a heterologous SHIV. This demonstration suggested that the antigenic cocktail vaccine strategy, which has succeeded in several other vaccine fields (e.g. pneumococcus), might also succeed against HIV-1. The strategy remains untested in an advanced clinical study, in part due to safety concerns associated with the use of replication-competent VV. To address this concern, we designed a macaque study in which psoralen/ultraviolet light-inactivated VV (UV VV) was substituted for replication-competent VV in the multi-envelope DVP protocol. Control animals received a vaccine encompassing no VV, or no vaccine. All VV vaccinated animals generated an immune response toward VV, and all vaccinated animals generated an immune response toward HIV-1 envelope. After challenge with heterologous SHIV 89.6P, animals that received replication-competent VV or UV VV experienced similar outcomes. They exhibited reduced peak viral loads, maintenance of CD4+ T cell counts and improved survival compared to control animals that received no VV or no vaccine; there were 0/15 deaths among all animals that received VV and 5/9 deaths among controls. Results define a practical means of improving VV safety, and encourage advancement of a promising multi-envelope DVP HIV-1 vaccine candidate.

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Disclosure Statement: The multi-envelope HIV-1 vaccine concept has been patented. A vector that may facilitate multi-envelope HIV-1 vaccine production has also been patented.

Keywords

HIV-1 vaccine; pathogenic SHIV; non-human primate; envelope cocktail; ultra violet-inactived vaccinia virus

INTRODUCTION

Despite important advances in the fields of immunology, virology and infectious diseases, no vaccine has yet matched the capacity of vaccinia virus (VV) to induce durable immunity and eradicate a human disease [1]. However, VV usage in humans has been associated with rare adverse events. The vaccine is not well controlled in immunodeficient patients or patients with excema, who may be exposed either by direct vaccination or by inadvertent transmission [2;3], and has been associated with myopericarditis [4]. Precautions can be taken to prevent adverse events, such as the use of subcutaneous (SQ) rather than percutaneous routes of vaccination [5], and the implementation of stringent training.

As one additional means of reducing risk, investigators have tested attenuated VV such as modified vaccinia virus Ankara (MVA)[6;7] [8], although some problems may be encountered during at the manufacturing stage due to slow growth. Another means of improving VV safety involves VV inactivation with psoralen and UV light [9]. This strategy can exploit (i) the robust growth characteristics of non-attenuated VV during manufacturing, and (ii) an inactivation methodology that has already been approved for clinical use [10–12].

The current study was designed to test a UV-inactivated VV product in the context of a multi-envelope, multi-vectored (DVP) HIV-1 vaccine. This vaccine was previously shown to control viral load and disease in macaques following a heterologous SHIV 89.6P challenge, even though no 89.6P envelope or any SIV component was represented by sequence in the vaccine [13–19]. A multi-envelope DVP vaccine was also tested in a brief clinical study in which it was both immunogenic and well-tolerated [16;20]. Cocktail vaccines have been successfully designed in other fields to combine mutually-exclusive membrane antigens and thereby recruit different populations of lymphocytes to function in unison to provide immune breadth. The study described here tested the UV-inactivation strategy in the context of DVP [14–16;19;21–23] as a means to improve safety and advance an attractive HIV-1 multi-envelope vaccine approach.

MATERIALS AND METHODS

Psoralen and UV inactivation of VV

VV were amplified on TK$^-$143B cells and purified by sucrose gradient sedimentation. Psoralen (UVADEX® methoxsalen, 10 μg/ml final concentration, Therakos, Inc., Eaton, PA) was added to viruses with preset titers of 2×10^7 – 2×10^8 pfu/ml. Human serum albumin was added to a final concentration of 0.1% and samples were incubated at room temperature for 10 minutes. Virus/psoralen mixtures were transferred to a polystyrene 6-well culture plate, 1 ml/well. Plates were placed in a Stratalinker 1800 (Stratagene, LaJolla, CA) and irradiated under 365 nm long-wave UV light in 3 minute intervals, with swirling between intervals, for a total of 12 minutes. Virus titers were determined by plaque assays on TK$^-$143B cells. Cells were stained with 10% Formalin/1% Methylene blue after 3 days of incubation for plaque enumeration. Plaques were only identified prior to UV treatments.

Macaque immunizations

Vaccine vector production has been described previously [24–27]. Delivery systems included recombinant DNA (D), recombinant VV (V) and recombinant protein (P). The

DNA vaccine was a combination of 52 different constructs, each expressing a different envelope protein (including envelopes from clades A,B,C,D and E) for a final total dose of 150 μg administered in a volume of 1.5 ml per animal by intramuscular (IM) injection. DNA was formulated either in PBS or in alum (500 micrograms per dose, Rehydrophos aluminum phosphate, Reheis, Inc.). The VV vaccine was a combination of 23 different constructs. VV was either replication competent (Live VV or Late VV) or UV inactivated (UV VV). Viruses were represented in equal quantities and the total dose per animal was 6.5×10^7 pfu in 1 ml delivered subcutaneously (SQ) between shoulder blades. The protein vaccine included four different purified envelope proteins from virus HIV-1_{1007} (clade B gp140, isolated in Memphis TN, purified by affinity chromatography from recombinant CHO cell supernatants), HIV-$1_{UG92005}$ (clade D gp140, purified by affinity chromatography from recombinant CHO cell supernatants), HIV-1_{MN} (clade B gp120, Protein Sciences Corp, Meriden, CT) and HIV-1_{CM} (clade E gp120, Protein Sciences Corp). The proteins were represented equally by weight, totaling 100 micrograms in 500 micrograms alum (Rehydrogel HPA, Reheis) in a 1 ml dose per animal, delivered IM. The majority of the proteins in the composite DVP vaccines were gp140 and a fraction of sequences were shared between the delivery vehicles. The SHIV 89.6P stock challenge virus was kindly provided by Drs. N. Letvin and K. Reimann [28]. Stock was diluted 1:1000 and delivered IV with 1 ml per animal (approximately 10 MID_{50}).

HIV-1 antibody assays

The HIV-1/2 Abbott ELISA (Abbott Laboratories, Abbott Park, Ill) was used. Antibody titers were calculated with curve-fitting software, defining the reciprocal serum dilution associated with an O.D. 492 nm reading of 0.1 (GraphPad Prism, San Diego, CA). Neutralization assays were performed as described previously [29]. To avoid false positives, serum immunoglobulin was purified on protein G columns and brought to its original sample volume prior to the preparation of serial dilutions. Percent neutralization was defined for test monkey samples by comparisons with a ‘no serum’ control.

Macaque SHIV virus loads and CD4 counts

To measure virus loads, branched DNA (bDNA) assays were performed by Bayer Reference Testing Laboratory (Berkeley, CA) on plasma samples. The CD4+ T cells were enumerated using Trucount kits (Becton Dickinson). Statistics were evaluated with GraphPad Prism Software using the Fisher’s Exact Test or Unpaired T Test as described in the text.

RESULTS

Inactivation of recombinant VV with psoralen and UV light

A first goal in the current study was to demonstrate that our recombinant VV could be inactivated with psoralen and UV light (Figure 1A). Following treatment, virus was incapable of producing plaques, but maintained capacity to express recombinant protein in tissue culture, confirmed by Western blot analyses. To ensure the replication incompetence of UV-inactivated VV, we injected product IP into SCID mice (Figure 1B). Ovaries were isolated 7 days later to test for plaque formation in vitro. Controls included replication competent viruses: (i) WRWT VV, (ii) non-recombinant NYCDH VV, and (iii) untreated HIV-1 envelope recombinant VV. The WRWT VV was used at a dose of 10^7 pfu (due to its high virulence), while the remaining viruses were used at a higher dose of 10^8 pfu. We found that the WRWT strain of VV was amplified to a much higher titer in ovaries compared to the NYCDH VV strain even though the original dose was ten-fold lower. The recombinant VV was further attenuated, and the UV-treated recombinant VV did not generate any plaques. When wildtype C57BL/6 mice were immunized twice with a DNA vaccine that expressed the clade D HIV-$1_{UG92005}$ envelope protein, followed by one VV

injection (either untreated or UV-inactivated VV expressing HIV-$1_{UG92005}$ envelope), anti-envelope antibody activity was boosted in all test mice (data not shown).

Recombinant UV-inactivated VV is immunogenic in macaques

The UV-inactivated VV was next tested in macaques. Animals were assigned to 5 groups (Figure 2A). The first four groups were immunized with multi-envelope vaccines designed to represent HIV-1 diversity as described previously [16;30;31], whereas the fifth group was not immunized. Vaccine delivery vehicles included recombinant DNA (D), recombinant VV (in some cases termed 'V') and recombinant protein (P), each formulated as cocktails to represent multiple HIV-1 envelope proteins [15;32]. Differences between groups are bolded in Figure 2A. The 'Live VV' Group received a DVP vaccine including two doses of replication-competent VV. The 'UV VV' Group received the same vaccine, but with UV/psoralen-inactivated VV. The 'Late VV' Group was the same as 'Live VV', except that there was only one dose of virus which was given relatively late in the regimen. This group was set up to test responses to replication-competent VV after multiple doses of DNA. The 'No VV' group received only DNA and protein. The 'No Vacc' group received no immunizations. One additional variable was that a portion of animals received DNA formulated in alum rather than PBS (indicated by '(A)' in animal names) to determine if alum would enhance DNA-based immune activities as described in previous literature [33].

Of note, all vaccines were well tolerated. All VV doses were administered by the SQ route and there were no cutaneous skin lesions in 'Live VV', 'UV VV' or 'Late VV' vaccinated animals [34]. To determine how virus inactivation affected the VV-specific antibody response, VV ELISAs were performed. VV-specific antibodies were detected in all animals in 'Live VV', 'UV VV' and 'Late VV' groups, but not in the 'No VV' group (Figure 2B). The first two groups exhibited similar antibody profiles suggesting that the UV-inactivation treatment did not significantly alter VV immunogenicity. Antibodies peaked after the second VV dose and then waned, but persisted throughout a 6 month period of evaluation. For 'Late VV' animals, the peak VV-specific antibody response was significantly reduced, perhaps because there was only one VV injection and because the anti-envelope responses induced by four previous DNA immunizations reduced the number and/or persistence of VV-infected cells [35].

Blood samples were also tested for HIV-1 envelope binding antibodies throughout the course of immunizations. As shown in Figure 3A-D, all vaccinated animals generated anti-envelope antibody responses and there were not significant differences between 'Live VV' and 'UV VV' animals, or between animals that received DNA in PBS or DNA in alum. Neutralizing assays were conducted with week 70 sera, taken one month prior to the SHIV challenge. Serially diluted samples were tested on target viruses including HIV-1_{IIIB}, HIV-1_{SF2} and the SHIV 89.6P. Neutralization scores of 50% or greater were considered positive. As shown in Figure 3E, all vaccinated animals neutralized HIV-1_{IIIB} and HIV-1_{SF2}, and there were sporadic responses toward SHIV 89.6P. Again, there were not significant differences between 'Live VV' and 'UV VV' animals.

Vaccine-induced protection against SHIV: reduced viral load

To examine the protective capacity of DVP in the context of 'Live VV', 'UV VV', 'Late VV' or 'No VV', animals were challenged intravenously with SHIV 89.6P. Viral loads were then monitored by bDNA analyses for several months. Peak viral loads were apparent two weeks after challenge as shown in Figure 4, panel A. The full time course of viral loads is shown in panels 4B-F. There were two outlier monkeys, EL28 in the 'UV VV' group and EM52 in the 'No Vacc' group that exhibited complete virus control. These animals are indicated by '#' in panel 4A and by inserts in panels 4C and 4F. The EL28 outlier result was

explained by a known technical difficulty experienced during the challenge procedure. EL28 results were therefore excluded from statistical analyses. For animal EM52, there was no known technical difficulty during the challenge procedure and the results were therefore included in statistical analyses. As shown in panel 4A, the highest peak viral loads were in the control 'No VV' or 'No Vacc' (no vaccine) groups. Several of these animals (6/9) exhibited peak viral loads that exceeded 1.5×10^8 copies per ml, while none of the animals that received 'Live VV', 'UV VV' or 'Late VV' experienced such high virus levels. The 'Live VV', 'UV VV', and 'Late VV' groups were not different, but each differed significantly from the 'No Vacc' group, despite exclusion of EL28 data (Fishers Exact test; $p<.05$). As shown in panels 4B-F, virus loads at later time points also trended lower for animals that received 'Live VV', 'UV VV' or 'late VV' compared to 'No VV' and 'No Vacc' controls, although statistically significant differences were not noted. Four animals that received VV (as well as the two outliers) achieved virus loads below 10^4 copies per ml as indicated by asterisks.

Vaccine-induced protection against SHIV disease: maintenance of CD4+ T cell counts

An additional analysis was of CD4+ T cell counts following challenge. As shown in Figure 5, animals that received VV ('Live VV', 'UV VV', or 'Late VV') maintained higher CD4+ T cell counts than the 'No VV' and 'No Vacc' control animals. The majority of 'No VV' and 'No Vacc' animals (5/9) showed CD4+ T cell counts that dropped below 1 cell/μl. All of these animals ultimately died as indicated by '+' marks in Figure 5. All animals that received a VV vaccine component maintained levels of CD4+ T cells above 1 cell/μl and survived. There were not significant differences between 'Live VV' and 'UV VV' groups. When groups were combined for analyses, those that received a VV component survived at a significantly higher rate than did control animals ('No VV' or 'No Vacc'; Fishers Exact Test $p<.05$). When examined independently, the 'Live VV' and 'UV VV' groups were also significantly better than controls in terms of CD4+ T cell maintenance and animal survival (Fishers Exact Test, $p<.05$). When the 'UV VV' group was compared directly to the 'No VV' group, CD4+ T cell numbers were yet again superior (week 17 values, unpaired T Test, $p<.05$).

DISCUSSION

The experiments described here were designed to address concerns related to the use of replication-competent VV in the context of a multi-envelope DVP vaccine. To improve safety, a clinically approved psoralen/UV light-inactivation technology was tested [36]. The treatment of VV by UV and psoralen may provide an advantage over virus attenuation, because attenuated virus growth poses difficulties at the manufacturing stage. In contrast, the strategy of VV inactivation occurs only after facile preparation of high-titered virus products.

Results in this report showed that 'UV VV' was comparable to 'Live VV' in terms of immunogenicity and protection in the context of DVP. In macaques that were vaccinated with multi-envelope DVP vaccines and challenged with SHIV 89.6P, the animals that received VV vaccines (Live VV, UV VV or Late VV) were better protected than controls (No VV or No Vacc) with regard to lower peak viral loads, CD4+ T cell maintenance, and animal survival.

All vaccines were well tolerated in the macaque study. VV vaccines were administered by the SQ route resulting in no cutaneous lesions even in the context of Live VV. The psoralen/UV light inactivation step provided an additional safety feature to support the continued use of VV in the clinical arena.

The success of the HIV-1 multi-envelope DVP vaccine was statistically significant, but full prevention of virus infection was not achieved. The reason for this limitation may relate, at least in part, to the origin of pathogenic SHIVs. When chimeric viruses were first produced to express HIV-1 envelope sequences with the SIV backbone, resultant viruses were not highly pathogenic. It was only after multiple monkey passages that SHIVs were isolated with greater monkey-tropism and pathogenicity [37], but this associated with mutations in the HIV-1 envelope. Immune responses elicited by natural HIV-1 envelopes may not be sufficiently cross-reactive with the mutated SHIV envelopes to prevent infection. Despite the limitations of the monkey model, our results demonstrated that a heterologous, multi-envelope vaccine devoid of SIV components could prevent disease and death following SHIV challenge.

For animals that initially control virus infection in the SHIV challenge model, why do some progress toward severe disease and others do not? This perhaps relates to the acute damage caused when peak viral titers are reached. Of the six animals in the current study that reached peak viral loads above 1.5×10^8 copies per ml, most (66%) ultimately suffered severe CD4+ T cell loss and death. Among the 18 animals that experienced peak viral loads below 1.5×10^8, there was only 1 death (<10%). Such associations between peak viral load and animal survival illustrate the attributes of vaccination, even in a monkey model in which full protection is difficult to achieve. The precise correlates of protection conferred by the DVP vaccine described in this report were not fully discerned. There are numerous mechanisms of antibody-mediated protection against virus that deserve further analyses, as well as the robust CD4+ and CD8+ T cell responses known to be induced by the DVP vaccine [38–44].

How can the full capacity of the immune system be harnessed? We propose that a HIV-1 vaccine should be used to pre-activate not just one type of lymphocyte [45;46], but a variety of lymphocytes with diverse receptor specificities, capable of recognizing different HIV-1 envelope structures. This strategy has been highly successful in other fields. The licensed *Pneumovax* encompasses 23 different components and vaccines of lesser complexity often associate with an increase in escaped bacterial serotypes [47;48]. The *Pneumovax* experience demonstrates the need to achieve a fine balance between vaccine simplicity and antigenic coverage when targeting a diverse pathogen. In the HIV-1 field, this balance may ultimately be achieved by advancing a multi-envelope vaccine approach.

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HIGHLIGHTS

- UV-inactivated vaccinia virus is a successful HIV-1 vaccine vehicle
- A multi-envelope HIV-1 vaccine protects against heterologous SHIV in macaques
- DNA-vaccinia virus-protein prime-boost vaccine protects against heterologous SHIV

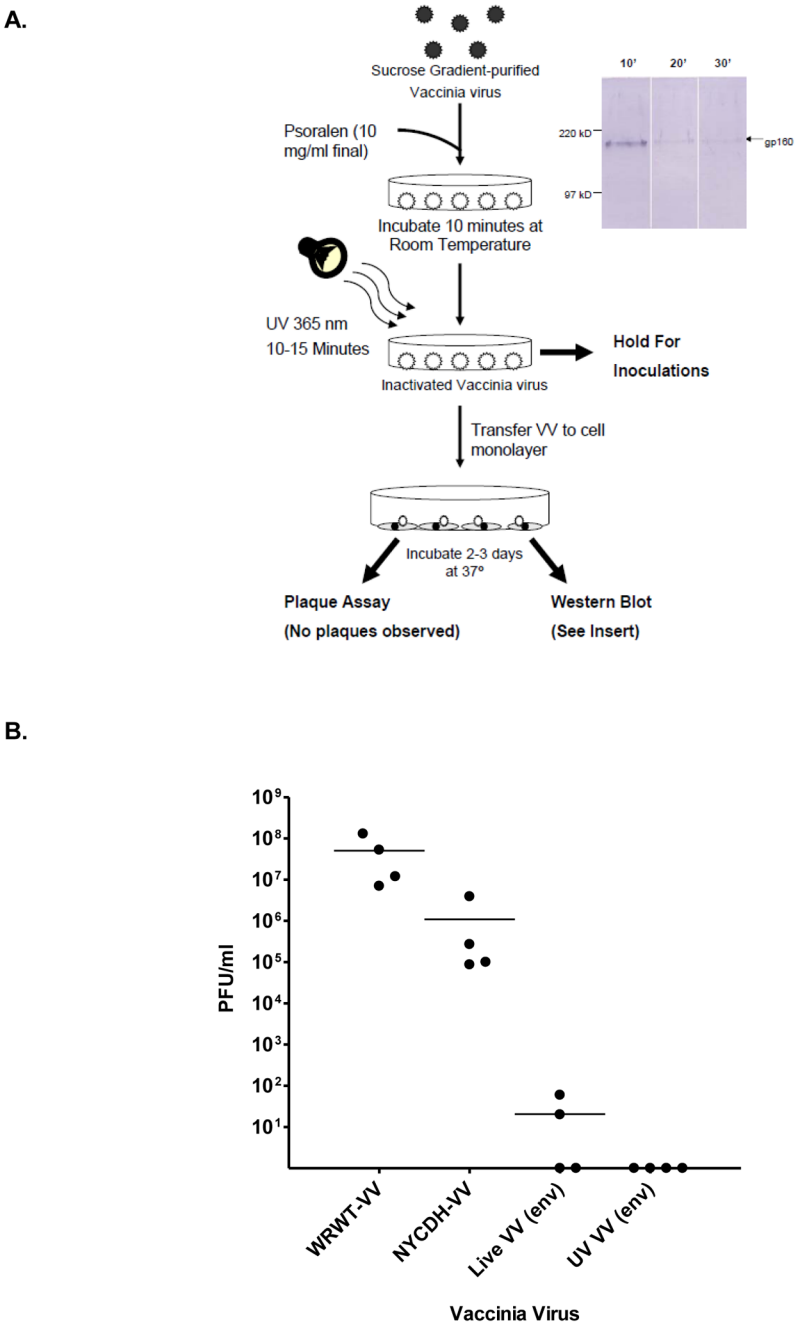


Figure 1. Inactivation of VV

Panel A. A flowchart of VV inactivation is shown. Virus was purified by sucrose gradient and then treated with psoralen and UV light. UV-treated virus was measured by plaque assay and Western blot to ensure inactivation and to ensure capacity for protein expression. Panel B. SCID mice were administered live VV of the WRWT strain (WRWT-VV, 10^7 pfu), live VV of the NYCDH strain (NYCDH-VV, 10^8 pfu), live recombinant NYCDH VV (Live VV (env),10^8 pfu) and inactivated live recombinant NYCDH VV (UV VV (env), originally 10^8 pfu). After 7 days, ovaries were assessed for plaque formation on mammalian cell monolayers. Results from individual mice and means are shown.

A.

Vaccine group	Live VV	UV VV	Late (Live) VV	No VV	No Vacc
Animals	EN08 (A) EM33 DE65 (A) DG35 DJ86 DJ95	EI76 (A) EL28 DL03 (A) DF48 DR22 EI80	BR66 DT12 (A) EB07	CA12 EJ60 (A) EK99	BG38 DK29 DR29 EF20 EJ18 EM52
Vaccinations					
Week 0	DNA	DNA	DNA	DNA	**None**
Week 4	DNA	DNA	DNA	DNA	**None**
Week 9	**Live VV**	**UV VV**	**DNA**	**DNA**	**None**
Week 24	**Live VV**	**UV VV**	**DNA**	**DNA**	**None**
Week 29	**DNA**	**DNA**	**Live VV**	**DNA**	**None**
Week 33	Protein+alum	Protein+alum	Protein+alum	Protein +alum	**None**
Week37	DNA	DNA	DNA	DNA	**None**
Week 41	Protein+alum	Protein+alum	Protein+alum	Protein+alum	**None**
Week 66	Protein+alum	Protein+alum	Protein+alum	Protein+alum	**None**
Week 70	DNA	DNA	DNA	DNA	**None**
Challenge-Week 74	SHIV 89.6P	SHIV 89.6P	SHIV 89.6P	SHIV 89.6P	SHIV 89.6P

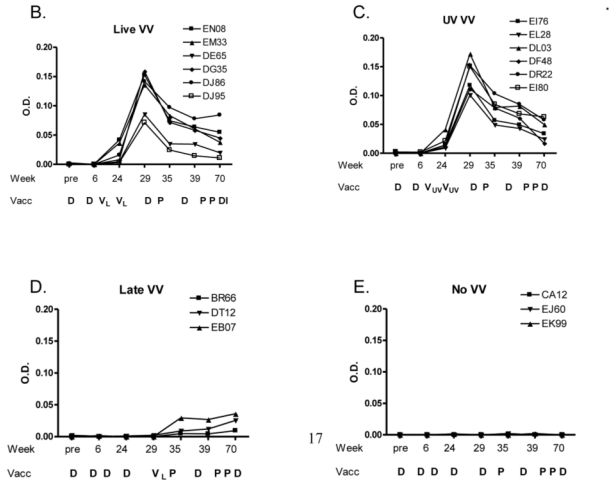
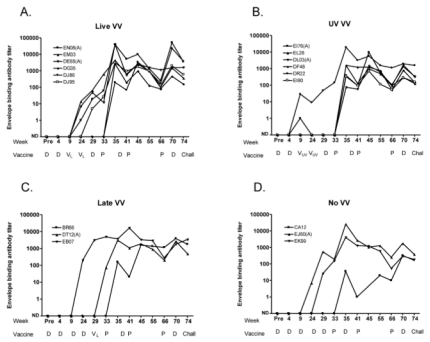


Figure 2. DVP elicits VV-specific antibody activities in macaques

Panel A. The schedule of monkey immunizations is shown. DNA (150 μg, 52 envelopes) was delivered IM in 1.5 ml PBS. An (A) designates an animal that received all DNA vaccines formulated in 500 μg alum rather than PBS. VV was delivered SQ (6.5×10^7 original pfu; 23 envelopes) and protein was delivered IM (100 μg in 500 μg alum, 4 envelopes). Bolded entries highlight differences between the monkey groups

Panels B-E. Macaques received a DVP vaccine (panels A, B, C) or a control vaccine including no VV (panel D). Vaccinations are shown below each graph. Groups received two doses of live VV (V_L, panel A), two doses of inactivated VV (V_{UV}, panel B), one dose of live VV (panel C), or no VV (panel D). ELISAs were conducted to monitor VV-specific antibody titers throughout the immunization scheme.



E.

Virus Strain:	HIV-1_{IIIB}			HIV-1_{SF2}			SHIV 89.6P	
Serum Dilution:	1:1000	1:100	1:10	1:1000	1:100	1:10	1:100	1:10
Live VV								
EN08 (A)	0	**99**	**97**	9	**80**	**100**	8	**52**
EM33	4	**90**	**90**	9	46	**98**	6	18
DE65 (A)	6	**92**	**100**	3	18	**99**	29	37
DG35	7	**100**	**100**	8	**64**	**100**	14	6
DJ86	8	**100**	**100**	14	**89**	**98**	0	12
DJ95	8	**100**	**100**	18	19	**98**	6	24
UV VV								
EI76 (A)	4	**99**	**90**	3	**68**	**99**	0	0
EL28	2	**91**	**100**	12	36	**98**	0	0
DL03 (A)	7	**100**	**100**	14	0	**94**	0	42
DF48	3	**100**	**100**	16	38	**98**	0	0
DR22	0	**100**	**100**	0	**97**	**99**	19	**88**
EI80	4	**100**	**97**	10	**72**	**99**	0	0
Late VV								
BR66	4	**96**	**100**	10	**51**	**96**	0	0
DT12 (A)	6	**100**	**100**	2	**82**	**96**	0	38
EB07	0	**87**	**95**	0	**95**	**96**	14	**50**
No VV								
CA12	0	25	**85**	18	20	**94**	0	0
EJ60 (A)	2	**100**	**100**	3	**70**	**97**	0	0
EK99	5	**95**	**95**	1	**79**	**97**	35	**56**
No Vac								
BG38	8	0	0	0	0	0	0	0
DK29	4	0	0	3	0	0	0	0
DR29	3	0	0	4	0	0	0	0
EF20	7	0	0	8	0	1	0	0
EJ18	10	0	0	8	0	0	0	0
EM52	0	0	0	0	0	0	0	0

Figure 3. DVP elicits HIV-specific antibody activities in macaques

Macaques received Live VV (panel A), UV VV (panel B), Late VV (panel C) or No VV (panel D). The Abbott ELISA was conducted with serially diluted serum samples to monitor envelope-specific antibody titers throughout the immunization scheme. Curve fitting software defined reciprocal serum dilutions associated with an O.D. reading of 0.1, graphed on a log scale. ND-No detected antibody. E. Neutralization was tested pre-challenge (week 70) against HIV-1_{IIIB} and HIV-1_{SF2} and SHIV 89.6P. Entries exceeding 50% were considered positive and were highlighted in bold.

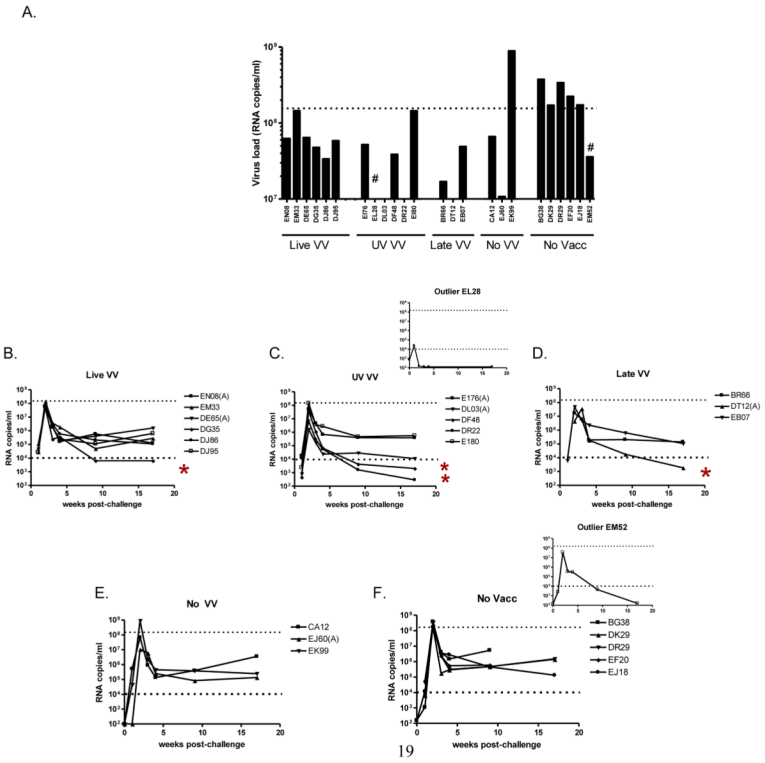


Figure 4. DVP protects macaques from SHIV challenge by reducing peak virus loads Peak virus loads are shown in panel A (RNA copies per ml, Bayer bDNA assay; samples taken 2 weeks after challenge)

'#' identifies outlier animals described in the text. A time course of virus loads is also shown following SHIV challenge for the five animal groups: Live VV (panel B), UV VV (panel C), Late VV (Panel D), No VV (Panel E) and No Vacc (Panel F). Inserts include data for outlier animals described in the text. Dotted lines highlight virus levels of 1.5×10^8 (panel A) and 1×10^4 (panels B-F) copies/ml to assist animal comparisons. Asterisks identify animals that were not outliers with levels lower than 10^4.

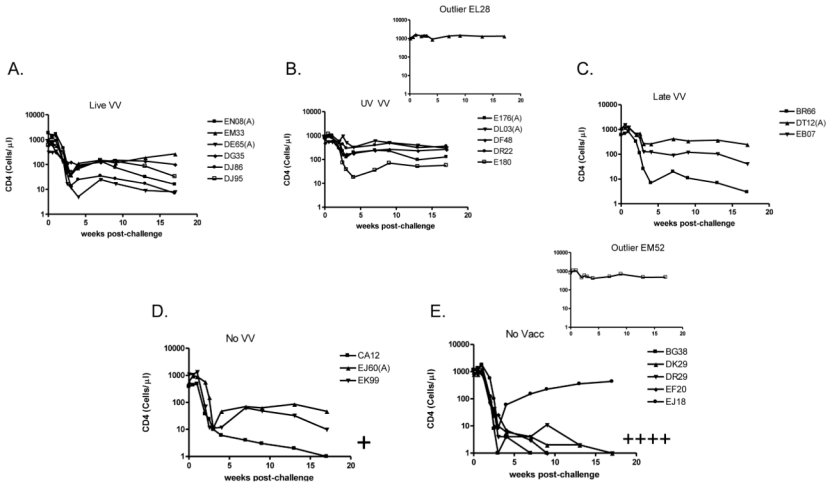


Figure 5. DVP protects macaques from SHIV challenge by controlling CD4+ T cell counts

CD4+ T cell counts/μl are shown throughout the course of immunizations for the five animal groups. Animals received Live VV (panel A), UV VV (panel B), Late VV (panel C), No VV (panel D) or No Vacc (panel E). Inserts include data for outlier animals described in the text. '+' indicates animals with CD4+ T cell counts <1 cell/μl; these animals subsequently died.