Published in final edited form as:

AIDS. 2016 October 23; 30(16): 2405–2414. doi:10.1097/QAD.0000000000001224.

Antibody responses to prime-boost vaccination with an HIV-1 gp145 Env protein and chimpanzee adenovirus vectors expressing HIV-1 gp140

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Abstract

Objectives—Over two million individuals are infected with human immunodeficiency virus type 1 (HIV-1) each year, yet an effective vaccine remains elusive. The most successful HIV-1 vaccine to date demonstrated 31% efficacy. Immune correlate analyses associated HIV-1 envelope (Env)-specific antibodies (Abs) with protection, thus providing a path toward a more effective vaccine. We sought to test the Ab response from novel prime-boost vaccination with a chimpanzee-derived adenovirus (AdC) vector expressing a subtype C Env gp140 combined with either a serologically distinct AdC vector expressing gp140 of a different subtype C isolate or an alum-adjuvanted, partially trimeric gp145 from yet another subtype C isolate.

Design—Three different prime-boost regimens were tested in mice: AdC prime-protein boost, protein prime-AdC boost, and AdC prime-AdC boost. Each regimen was tested at two different doses of AdC vector in a total of 6 experimental groups.

Methods—Sera were collected at various timepoints and evaluated by ELISA for Env-specific Ab binding, isotype, and avidity. Ab functionality was assessed by pseudovirus neutralization assay.

Results—Priming with AdC followed by a protein boost or sequential immunizations with two AdC vectors induced HIV-1 Env-specific binding Abs including those to the V2 region, while priming with protein followed by an AdC boost was relatively ineffective. Abs that cross-neutralized tier 1 HIV-1 from different subtypes were elicited with vaccine regimens that included immunizations with protein.

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Disclaimers/Conflicts of Interest Statement: The Ertl and Polonis Labs declare no conflicts of interest. The views expressed are those of the authors and should not be construed to represent the positions of the U.S. Army or the Department of Defense.

Conclusions—Our study warrants further investigation of AdC vector and gp145 protein prime-boost vaccines and their ability to protect against acquisition in animal challenge studies.

Keywords

HIV-1; vaccine; antibodies; envelope proteins; neutralization; adenovirus vector

INTRODUCTION

Thirty-five million people are living with human immunodeficiency virus (HIV) and over 2 million become infected each year. Despite advances in therapy, HIV causes over 1.5 million deaths annually. A vaccine to prevent HIV type 1 (HIV-1) infection could drastically reduce the global burden of disease. A desirable immune target is the trimeric HIV envelope (Env) glycoprotein that facilitates entry into susceptible host cells, which can be blocked by antibodies (Abs).

The complexity of the virus presents challenges for an efficacious vaccine. There have been six HIV-1 vaccine efficacy trials to date[1–5]. The RV144 trial, which tested priming with a canarypox vector expressing Gag, Pol, and Env, and boosting with monomeric gp120 protein, resulted in 31.2% efficacy[6]. This offered the first indication that a vaccine could prevent HIV-1 infection, albeit with modest and transient efficacy. IgG Abs, specifically IgG3 targeting the variable region 2 (V2) of gp120, correlated with decreased risk of infection[7–9].

Adenovirus (Ad) vectors of human serotype 5 are potent inducers of T and B cell responses, but failed in HIV-1 vaccine trials, in part due to pre-existing immunity to the vaccine vector[10]. Vaccines based on alternative Ad viruses, with lower seroprevalence in humans such as those derived from chimpanzees (AdC), might be more advantageous[11]. Several AdC vectors have been shown to be highly immunogenic in mice and nonhuman primates[12,13]. In addition, prime-boost regimens based on AdC7 and AdC6 demonstrate induction of potent and sustained transgene-specific T and B cell responses[14].

Trimeric HIV-1 Env may elicit improved immune responses compared to monomeric gp120, as it may be more representative of a functional Env trimer on the surface of the virion[15]. Several trimeric Env proteins have recently been shown to elicit neutralizing Abs (NAbs) in preclinical studies[16–18]. The CO6980 gp145 Env, isolated from an acute, subtype C infection, represents a tier 2 HIV-1 envelope with an intact membrane-proximal external region extended by 3 lysine residues that binds to integrin $\alpha 4\beta 7$ and several monoclonal Abs (mAbs), including V2 mAbs. Immunization of rabbits elicited binding Abs to Env, including V1V2 and V3 specificities, and cross-reactive NAbs[16].

We tested AdC vectors and recombinant protein in prime-boost vaccine strategies, utilizing replication-defective AdC vectors expressing subtype C Env without the transmembrane domain (gp140), and subtype C gp145 protein, CO6980[16]. We assessed the immunogenicity of six prime-boost regimens, categorized as three prime-boost strategies tested at two AdC vector doses. The first strategy involved priming 3 times with gp145 protein followed by boosting with AdC vector, the second primed once with AdC vector

then boosted 3 times with gp145. The third primed once with AdC vector then boosted once with a heterologous AdC vector.

Boosting AdC vector-primed mice with the heterologous AdC vector or alum-adjuvanted gp145 protein significantly enhanced Env-specific Ab responses including V2-specific binding Abs. Priming with protein followed by AdC vector boost was relatively ineffective. Induction of NABs that cross-reacted between different clades required inclusion of protein into the vaccine regimen. This study warrants further investigation of AdC vector and gp145 protein prime-boost vaccines.

METHODS

Vaccines

Ad vectors based on chimpanzee serotypes SAd-V24 (AdC7) and SAd-V23 (AdC6) were E1-deleted, generated from recombinant viral molecular clones, and encode HIV-1 subtype C gp140 transgenes, strain Du172 and Du422, respectively. Vectors were produced, purified, titrated and quality controlled as described[13].

Construction and production of gp145 CO6980 Env protein subtype C have been described[16]. Briefly, CO6980 Env was expressed in CHO-K1 cells and purified by lectin affinity chromatography with *Galanthus nivalis* lectin agarose (Vector Laboratories, Burlingame, CA) followed by anion exchange on Q-Sepharose fast flow (GE Healthcare, Little Chalfont, UK). Protein was further concentrated, exchanged into phosphate-buffered saline (PBS) and sterile filtered. Oligomeric form was determined to be about 60% trimeric by negative stain electron microscopy.

Vaccination protocol

Female outbred Crl:CD1(ICR) mice were treated at the Wistar Institute Animal Facility following approved procedures. Vaccines were administered intramuscularly in 200 μ L/dose. AdC gp140 vectors were given in PBS; gp145 protein was given at 10 μ g/dose in a 1% aluminum hydroxide gel suspension (alum; Sigma-Aldrich).

Serum isolation

Serum prepared from whole blood was frozen at -20°C .

IgG ELISA

Sera were tested for HIV-1 gp140-specific and V2-specific Abs by ELISA on plates coated with gp140 protein or linear V2 peptide, respectively. HIV-1 strain Du172 gp140 protein was produced in baculovirus[19]. V2 peptide was based on a 19 amino acid immunodominant linear epitope within V2 of HIV-1 strain Du172: TTTEITDKKKKEYALFYKL (Genscript, Piscataway, NJ)[20]. Gp140-specific IgG was tested in 2-fold serum dilutions from 1:1000–1:16,000. V2-specific IgG was tested using 2-fold serum dilutions from 1:100–1:6400. Briefly, 96-well plates (Nunc-Immuno™) were coated overnight at 4°C with (A)gp140 protein (200ng/well) or (B)V2 peptide (400ng/well). Wells were blocked overnight at 4°C with (A)PBS/3% BSA/0.1% Tween-20 or (B)PBS/8% BSA/0.1% Tween-20. Duplicate

samples and a standard were incubated at RT for (A)2h or (B)90 min. Bound IgG was detected with goat anti-mouse IgG alkaline phosphatase conjugate (Sigma-Aldrich) at (A)1:30,000 or (B)1:15,000. Bound enzyme was detected with phosphatase substrate tablets (Sigma-Aldrich) in DEA substrate buffer (KPL) by optical density (OD) at 405 nm. Gp140-specific IgG is reported as area under the curve (AUC) for 1:2000–1:16,000 dilution curves. V2-specific IgG is reported as OD at 1:100 dilution.

Sub-isotype ELISA

Sub-isotyping was performed similarly as above except bound Ab was detected using rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM (Calbiochem) and goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma-Aldrich). Gp140-specific and V2-specific sub-isotypes were tested at serum dilutions of 1:2000 and 1:200, respectively.

Avidity ELISA

Avidity of gp140-specific Abs was assessed by NaSCN-displacement ELISA. Briefly, samples were diluted to 1:2000 in PBS/3% BSA/0.1% Tween-20 and incubated for 2h at RT on 96-well plates (Nunc-Immuno™ Microwell™) coated with HIV-1 gp140 strain Du172. Ascending concentrations of the chaotropic agent NaSCN were added to the wells (0–4 M). Plates were incubated for 15 min at RT then treated with goat anti-mouse IgG alkaline phosphatase conjugate (Sigma-Aldrich) at 1:30,000. Bound enzyme was detected as above by OD at 405 nm.

Pseudovirus preparation

Pseudovirus (PV) was prepared as previously described[21]. The following PVs were produced: GS015 and MW965, both tier 1 subtype C; TH023, tier 1 CRF01_AE; SF162, tier 1 subtype B; TZBD 9/11, tier 2 subtype C; and murine leukemia virus (MuLV) (nonspecific control).

High-throughput PV neutralization assay

Neutralizing antibody titers were determined using TZM-bl cells in a high-throughput assay utilizing robotic liquid handling. Serum was diluted 1:10 in growth medium and serially diluted using the Biomek NXP liquid handler (Beckman Coulter, Indianapolis, IN)[22]. Titered serum (12.5 μ L/well) was transferred to 384-well culture plates and incubated with an equal volume of PV for 45 min at 37 °C. TZM-bl cells (3×10^3 cell/well) with DEAE-dextran (25 μ g/mL) were added to each well and incubated for an additional 48 hours. Relative light units (RLU) were detected with the SpectraMax Paradigm Microplate Reader (Molecular Devices, Sunnyvale, CA) using the Bright-Glo Luciferase Assay System (Promega Corporation, Madison, WI). Percent neutralization (percent reduction of RLU in the presence of serum) was calculated for each serum dilution. Neutralization dose-response curves were fitted by nonlinear regression using the LabKey Server, and the final titer is reported as the reciprocal of the dilution of serum necessary to achieve 50% neutralization (50% inhibitory dose [ID₅₀]).

Statistical analysis

One-way ANOVA was used with Holm-Sidak correction for comparing three or more parameters for one group or one parameter for three or more groups. Two-way ANOVA was used with Holm-Sidak correction for comparing multiple parameters among three or more groups. Analyses were performed in GraphPad Prism 6.

RESULTS

Study Design and Vaccines

The study design is shown in Fig. 1A. Groups 1 and 2 tested protein prime at day 0, week 4, and week 8 followed by a boost with 10^{10} or 10^9 vp of AdC7 at week 12. Groups 3 and 4 tested AdC prime at the 2 doses followed by protein boosts at weeks 8, 12, and 18. Groups 5 and 6 tested priming with 10^{10} or 10^9 vp of AdC7 at day 0 and boosting with heterologous AdC6 using the same doses at week 8. Sera were collected at the indicated time points.

Magnitude and Kinetics of Ab Responses

Ad vectors are very immunogenic and induce both B cell and T cell responses[10,13,14]. These responses can be boosted by heterologous vaccines, such as a protein or an Ad vector from a different serotype. We sought to determine which of the six immunization regimens elicited the highest and most sustained HIV-1 Env-specific Ab response. In addition, the study was designed to assess if any of the regimens allowed for dose sparing of the AdC vector.

Binding Ab responses to gp140 Env strain Du172 subtype C are shown for individual mice (Fig. 1B, 1C) and for mean response by group (Fig. 1D, 1E). We sought to determine (1) whether the prime-boost regimen increased Ab titers over baseline response (wk 0), (2) whether boosting increased Ab titers over that induced by the prime, and (3) whether Ab titers were sustained. For this analysis, Ab titers were compared within each group at all timepoints (weeks 0 through 24). In the AdC7/protein 10^{10} group there was a significant increase in Ab titers from baseline beginning at week 4 and all the timepoints thereafter (*p* values reported in the figure legend and Supplement Table 1). In the AdC7/protein 10^9 group, a significant increase in titers from baseline was first seen after the protein boost (wk 12) and was maintained thereafter. For both doses of AdC7/protein there was a significant increase from responses 4 and 8 weeks after AdC7 prime and all timepoints after the protein boosts. However, for both AdC7/protein groups there were no significant differences among Ab responses at weeks 12, 18, 20, and 24, indicating that the second and third protein boosts did not increase titers. For both doses of AdC7/AdC6 a significant increase in titers from baseline was first seen after the AdC6 boost. There was a significant increase from the response 4 weeks after AdC7 prime to all timepoints following the AdC6 boost. In addition, the 10^9 vp AdC7/AdC6 group showed a difference between week 8 after AdC7 prime, the timepoint immediately prior to AdC6 boost, and week 24. In the protein/AdC7 10^{10} group a significant increase in titers from baseline was seen after the AdC7 boost. An increase in titers from week 4 was seen following the AdC7 boost. There were no significant differences in Ab titers over baseline or among timepoints in the protein/AdC7 10^9 group.

We sought to determine if one type of prime-boost regimen offered an advantage over the others. We compared the different regimen types at each dose of AdC. When comparing the three regimens at the 10^{10} vp dose of AdC (Fig. 1D), the AdC7/protein regimen elicited the highest titers of binding Abs. Ab responses to the AdC7/protein regimen were significantly greater than those to the protein/AdC7 regimen by 8 weeks after priming ($p=0.0204$) and significant differences were maintained through week 24. AdC7/protein induced higher responses than AdC7/AdC6 by 4 weeks after the first boost, which were sustained through week 24. There was a marginally higher response in AdC7/protein compared to AdC7/AdC6 at week 8 ($p=0.0451$) despite receiving the same prime, however after boosting the difference was highly significant (wk 12 $p<0.0001$). There were no significant differences between the protein/AdC7 and AdC7/AdC6 regimens. When comparing the three regimens at the 10^9 vp dose of AdC (Fig. 1E), the AdC7/protein-induced Ab response was significantly greater than that elicited by protein/AdC7 from weeks 12 through 24. The AdC7/AdC6 response was greater than the protein/AdC7 response only at week 24. There were no significant differences in responses when comparing the AdC7/protein and AdC7/AdC6 regimens at the 10^9 vp dose.

We were interested in ascertaining whether dose sparing of the AdC vector is possible. Thus, we compared the two doses (10^{10} vp AdC and 10^9 vp AdC) for each regimen type. In the AdC7/protein regimens, Ab response were significantly higher in the 10^{10} vp group as compared to the 10^9 vp group at 8 weeks post-prime ($p=0.0191$), differences observed at other time points were not significant. For both the protein/AdC7 regimens and the AdC7/AdC6 regimens, there were no differences between the 10^{10} and 10^9 vp groups.

As we showed previously, Ab responses induced by Ad vectors are extremely stable, which may reflect the low level in vivo persistence of Ad vectors[23,24]. We tested some of our mice 46 weeks after priming and their titers were comparable to those tested earlier at week 18. Using a different monomeric gp140 for a single boost of an AdC-induced Ab response showed that responses, once they peaked around week 18, remained stable through week 46 (data not shown).

Overall these results show that boosting of an AdC vector with a serologically distinct AdC vector or an alum-adjuvanted gp145 protein significantly enhances Ab responses. Interestingly, repeated immunizations with the gp145 protein did not significantly increase Ab responses nor did it seem to affect response longevity.

We further characterized the Ab response by isotype for all regimens (Fig. 2A,B). For groups primed with the AdC vector, the Ab response was primarily composed of IgG2a (55–65%) and IgG2b (25–36%) isotypes, indicative of responses driven by type 1 T helper (Th1) cells. We also detected IgG1 (3–11%) in these groups, an isotype that is promoted by type 2 T helper (Th2) cells, but little to no IgG3, IgM, or IgA. In mice that were primed with protein approximately half of the Ab response was comprised of IgG1 (44%), while IgG2a and IgG2b responses were comparable (22–30%) and we detected some IgG3, IgM and IgA (0.5–3%). Comparing the groups within a given AdC vector dose showed significant differences in IgG1 and IgG2a between protein/AdC7 compared to AdC7/protein or AdC7/AdC6 ($p<0.0001$). These data show that priming with the AdC vectors induced

predominantly a Th1 response and that this was not modified by boosting with aluminum hydroxide-adjuvanted protein, which generally induces a Th2 response[25,26]. Priming with protein, on the other hand, favored induction of a Th2 response.

Ab Function

We tested the avidity of the Env-specific Abs induced by the vaccine regimens at the week following the last immunization (Fig. 3A). The groups that received the protein immunizations first showed markedly lower avidity compared to the others and only retained 50% antibody binding at NaSCN concentrations slightly above 1M. The other groups retained 50% Ab binding at 2M NaSCN or greater (Fig. 3A). To determine the effect of additional protein boosts on Ab avidity in the AdC7/protein 10^{10} and AdC7/protein 10^9 groups, samples from week 12, 18 and 20 were evaluated (Fig. 3B). There were no significant differences in the avidity after additional protein boosts.

To assess a virus inhibitory function of the antibodies, we performed TZM-bl neutralization assays against 4 tier 1 PVs, MW965 (subtype C), GS015 (subtype C), SF162 (subtype B) and TH023 (subtype CRF01_AE), and 1 tier 2 PV, TZBD 9/11 (subtype C) (Fig. 4). A PV containing the Env from murine leukemia virus (MuLV) was tested as a non-specific control. Sera were tested at different times after vaccination and the ID_{50} values were determined. Sera failed to neutralize MuLV (not shown). All of the vaccine regimes induced neutralizing antibodies to MW965 PV, with the highest titers observed in the group receiving AdC7/protein 10^{10} . Response rates to this virus, shown as the ratio of seropositive mice over all mice in a given group (Suppl. Fig 1), were comparable between all 6 groups (0.8) by the end of the study. Regimens that included immunizations with protein also induced cross-clade NABs to other tier 1 viruses, although response rates were lower. Responses to the two AdC vectors were less broad. None of the regimens induced NABs to the tier 2 subtype C virus.

V2-specific Ab Response

We tested whether linear V2-specific Abs, shown to be protective in the RV144 trial were elicited by our vaccine regimens (Fig. 5A)[7]. Anti-V2 Abs were detected in all vaccine groups. In the AdC7/protein 10^{10} and AdC6/AdC7 10^{10} groups there were significant increases in V2-specific Ab titers from baseline ($p<0.0001$ and $p=0.0255$, respectively). We characterized the V2-specific response by isotyping (Fig. 5B). The AdC7/protein V2-response was dominated by IgG2a antibodies, while the other groups showed a more even distribution of IgG1, IgG2a and IgM. IgM was overall higher than in the response to whole protein (Fig. 1), suggesting that the response to the V2 loop matures with a delay.

DISCUSSION

AdC vaccines are being developed against several infectious diseases, including HIV-1, due to their low seroprevalence in humans compared to human Ad, their high immunogenicity, and ease of modification[23–25]. The HIV-1 CO6980 trimer-enriched gp145 Env immunogen elicits advantageous Ab responses in rabbits and is undergoing GMP production for use in human clinical trials[16]. In this study, immunogenicity of novel vaccine regimens involving AdC vectors expressing a subtype C gp140 and heterologous CO6980 gp145

protein were tested in mice. The goal was to assess Ab responses elicited by three different prime-boost regimens (protein/AdC7, AdC7/protein, and AdC7/AdC6) at two different doses of AdC vector.

AdC7/protein regimen elicits significantly higher IgG titers compared to the protein/AdC7 and AdC7/AdC6 regimens at certain doses. These results are consistent with other studies that demonstrate optimal immune responses from heterologous prime-boost vaccination, when protein is used as the boost [26–28]. The mechanism for higher Ab responses upon protein boost is not fully understood. We would assume it reflects that AdC vectors are by far more immunogenic than protein vaccines and are thereby better suited to drive activation of naïve B cells, which can then be expanded by a less potent vaccine. However, further protein boosting did not significantly increase Ab titers. It was also not essential to ensure durability of the response as we tested for using a different gp140 protein for boosting (unpublished data). This suggests that heterologous protein boosts may be required or that a single protein boost may suffice after an AdC vector prime. The latter would be highly advantageous considering the cost of protein vaccines and the complexity of multiple dose regimens.

All of the vaccine regimens induced Env-specific IgG2b which is the mouse isotype most closely related to IgG3 that was shown to correlated with vaccine-induced resistance to HIV-1 infection in the RV144 trial [29]. Our results differ from the IgG1 biased response in a protein and DNA immunizations protocol [30].

Ab avidity was evaluated to determine the strength of Ab binding as an assessment of affinity maturation. Previous studies showed an inverse correlation between post challenge viral titers and the avidity of Abs induced by preventative vaccination [31]. Our study did not find significant differences in Ab avidity among the AdC7/protein and AdC7/AdC6 regimens but markedly lower avidity in mice that received the protein primes followed by an AdC7 boost.

Induction of potent, broadly cross-reactive NAb is fervently pursued by HIV-1 vaccine researchers. They face many challenges, including extraordinary diversity of circulating strains, a high degree of glycosylation, and new epitopes revealed upon receptor and co-receptor binding. Large-scale clinical vaccine trials have induced low-titer NAb with limited cross-reactivity [1,2,6]. Our vaccine regimens that included protein immunizations induced Abs that were able to neutralize tier 1 viruses from subtype B, C and CRF01_AE, although they failed to neutralize the one tier 2 virus tested. NAb responses to the AdC7/AdC6 immunization were less broad, which may reflect that the structure of viral transgene products is distinct from that of a trimeric protein immunogen. Notwithstanding, including an AdC vector into a vaccine regimen has the added advantage that all Ad vectors induce potent cellular immune responses (most notable CD8⁺ T cells) to the transgene product, which may not prevent infection but allows for rapid viral clearance[32,33]. Other pre-clinical vaccine studies have also elicited NAb; tier 1 and tier 2 PV neutralization was achieved with a DNA vaccine expressing gp160 given together with particles displaying the membrane proximal external region of Env [34]. Similar results were obtained with an adjuvanted DNA vaccine followed by a protein boost, as well as with a DNA prime/protein

boost regimen using constructs that expressed diverse polyvalent heterotrimeric Envs [35,36]. Careful design of immunogens and adjuvants thus allows for the induction of cross-reactive NAbS to HIV-1, which upon passive transfer have been shown to protect nonhuman primates against challenge with an HIV-1/simian immunodeficiency virus (SIV) chimera and to reduce viral loads in chronically infected individuals in human trials [29,30,37].

In the RV144 trial, protection was correlated with Abs to the V2 region of gp120[7]. Subsequent studies confirmed these results in a nonhuman primate low dose SIV challenge study[32]. The AdC7 vector followed by AdC6 or protein boost also induced V2-specific Abs. This is in contrast to studies based on immunization with a monomeric gp120 protein[33].

Altogether our results are very promising as they show that a simple vaccine regimen can elicit cross-neutralizing (albeit tier 1 responses), as well as V2-specific antibodies in animals. Further studies are warranted in nonhuman primates to elucidate appropriate dosing of both the AdC7 vector and gp145 protein and to assess if Abs provide protection against viral challenge.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The Polonis Lab provided the gp145 protein (expressed and purified at ABL, Inc.) and pseudoviruses. The Ertl Lab generated the AdC vectors. S. Tuyishime performed immunizations and sera collections. K. Emmer performed sera collection, ELISAs, and statistical analyses. L. Wiczorek and S. Molnar performed pseudovirus neutralization assays and statistical analyses. K. Emmer, L. Wiczorek, H. Ertl, and V. Polonis contributed to the manuscript preparation.

Sources of funding: This work was supported by a grant from the NIH NIAID (5 U19 AI074078-05) (H.C.J.E.). This work was also supported by a cooperative agreement (W81XWH-11-2-0174) between the Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc., and the U.S. Department of Defense (DOD) (V.R.P.).

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Fig. 1. Vaccination with AdC expressing gp140 and partially trimeric gp145 protein induces strain Du172 gp140-specific Abs

Groups of 5 outbred Crl:CD1(ICR) mice were bled and immunized at the timepoints indicated in A. On immunization days mice were bled prior to vaccine administration and sera were isolated. Protein (P): gp145 protein strain C06980 10 μ g in 1% alum; AdC7 (C7): encodes gp140 strain Du172; AdC6 (C6): encodes gp140 strain Du422. Responses over time for individual mice in each group (B and C). Mean responses $\pm$ standard error of the mean (SEM) for each group (D and E). B and D show responses to the 10^{10} vp dose of AdC vectors, C and E show those to the 10^9 vp dose. Magnitude of IgG binding responses at each time point were determined by ELISA, calculated using background-adjusted optical density dilution curves, and are reported as area under the curve. All statistical comparisons were made by two-way ANOVA and Holm-Sidak multiple comparisons test; multiplicity adjusted *p* values are reported. The following significant adjusted *p* values were obtained:

Comparing response over time within each group:

(D) AdC7/protein 10^{10} : wk 0 vs. wks 4 – 24 $p < 0.0001$ – $p = 0.0009$, wk 4 vs. wks 12 – 24 $p < 0.0001$, wk 8 vs. wks 12 – 24 $p < 0.0001$ – $p = 0.0014$; AdC7/AdC6 10^{10} : wk 0 vs. wks 12 – 24 $p < 0.0001$, wk 4 vs. wks 12 – 24 $p = 0.0034$ – 0.0088 ; protein/AdC7 10^{10} : wk 0 vs. wks 18 – 24 $p = 0.0018$ – 0.0044 , wk 4 vs. wks 18 – 24 $p = 0.0079$ – 0.0174

(E) AdC7/protein 10^9 : wk 0 vs. wks 12 – 24 $p < 0.0001$, wk 4 vs. wks 12 – 24 $p < 0.0001$, wk 8 vs. wks 12 – 24 $p < 0.0001$; AdC7/AdC6 10^9 : wk 0 vs. wks 12 – 24 $p < 0.0001$ – $p = 0.0003$, wk 4 vs. wks 18 – 24 $p = 0.0005$ – 0.0078 , wk 8 vs. wk 24 $p = 0.0381$

Comparing different regimens at same dose:

(D) AdC7/protein 10^{10} vs. protein/AdC7 10^{10} : wks 8 – 24 $p < 0.0001$ – $p = 0.0204$; AdC7/protein 10^{10} vs. AdC7/AdC6 10^{10} : wk 8 $p = 0.0451$, wks 12 – 24 $p < 0.0001$ – $p = 0.0025$
(E) AdC7/protein 10^9 vs. protein/AdC7 10^9 : wks 12 – 24 $p = 0.0003$ – 0.0034 ; AdC7/AdC6 10^9 vs. protein/AdC7 10^9 : wk 24 $p = 0.0436$
Comparing AdC dose for same regimen:
AdC7/protein 10^{10} vs. AdC7/protein 10^9 : wk 8 $p = 0.0191$

Fig. 2. Ab responses are primarily IgG2

Isotyping was performed by ELISA following the last immunization: protein/AdC7 groups at wk 18, AdC7/protein groups at wk 20 and AdC7/AdC6 groups at wk 12. (A) At both AdC doses, AdC7/protein elicits a greater combined isotype response than protein/AdC7 and AdC7/AdC6 (10¹⁰ dose $p=0.0013$, $p=0.0034$, respectively; 10⁹ dose $p=0.0085$, $p=0.0442$, respectively). (B) Relative isotype frequency. Priming with protein versus AdC7 elicits a different isotype response independent of dose. Protein/AdC7 elicits predominantly IgG1 at a significantly greater frequency than both AdC7/protein and AdC7/AdC6, $p<0.0001$. Conversely the AdC7-primed regimens, AdC7/protein and AdC7/AdC6, predominantly elicit IgG2a and at greater frequency than protein/AdC7, $p<0.0001$. The symbols x/x over part B represent the significance differences between groups for the isotypes IgG1 and IgG2a, respectively (two-way ANOVA with Holm-Sidak multiple comparisons test).

Fig. 3. Avidity of Env-specific Ab binding

NaSCN-displacement ELISA performed (A) following last immunization: protein/AdC7 groups at wk 18, AdC7/protein groups at wk 20 and AdC7/AdC6 groups at wk 12 and (B) on week 12, 18, 20 for AdC7/protein 10^{10} and AdC7/protein 10^9 to determine effect of additional protein boosts on avidity. Results are reported as the concentration of NaSCN (mean $\pm$ SEM) at which IgG binding was reduced by 50% as compared to binding in the absence of NaSCN. (A) Avidity of AdC7/protein 10^{10} is significantly greater than both protein/AdC7 10^{10} and AdC7/AdC6 10^{10} , $p=0.0009$ and $p=0.0439$, respectively, while AdC7/AdC6 10^{10} is greater than protein/AdC7 10^{10} , $p=0.0339$. At the 10^9 dose, AdC7/protein and AdC7/AdC6 avidities are significantly greater than that of protein/AdC7, $p=0.0030$ and $p=0.0164$, respectively. (B) No significant change in avidity is seen in the AdC7/protein groups with administration of additional protein boosts. (all statistics performed by one-way ANOVA with Holm-Sidak multiple comparisons test).

Fig. 4. Breadth and potency of pseudovirus neutralization

Sera were screened at a 1:40 dilution against six PVs: MW965 and GS015 PVs (tier 1, subtype C), TH023 PV (tier 1, CRF01_AE), SF162 (tier 1, subtype B), TZBD PV (tier 2, subtype C), and MuLV PV (non-specific control) for all timepoints; 50% neutralization is the negative cut-off value (indicated by the dashed line). Statistical comparisons were made by two-way ANOVA and Holm-Sidak multiple comparisons test; significant multiplicity adjusted *p* values are reported. The x/x/x symbols within the graphs indicate significant differences with – symbolizing *p* values >0.05, * *p* values between 0.01 – 0.05 and ** *p* values between 0.001–0.01. The symbols are arranged from top to bottom to compare (top) protein/AdC7 to AdC7/protein, (middle) protein/AdC7 to AdC7/AdC6, (bottom) AdC7/protein to AdC7/AdC6.

More specifically the following difference reached significance with the following *p* values: MW965 PV: at wk 18 AdC7/protein 10^{10} > protein/AdC7 10^{10} $p=0.0258$, AdC7/protein 10^{10} > AdC7/AdC6 10^{10} $p=0.0317$; GS015 PV: protein/AdC7 10^{10} > AdC7/protein 10^{10} at wk 20 $p=0.0340$, protein/AdC7 10^{10} > AdC7/AdC6 10^{10} at wk 20 $p=0.0102$ and wk 24 $p=0.0424$, AdC7/protein 10^9 > protein/AdC7 10^9 at wk 24 $p=0.0032$, AdC7/protein 10^9 > AdC7/AdC6 10^9 at wk 24 $p=0.0057$; TH023 PV: at wk 12 AdC7/AdC6 10^9 > protein/AdC7 10^9 $p=0.0073$, AdC7/AdC6 10^9 > AdC7/protein 10^9 $p=0.0115$; SF162 PV: protein/AdC7 10^{10} > AdC7/AdC6 10^{10} at wk 20 $p=0.0318$, protein/AdC7 10^9 > AdC7/protein 10^9 at wk 12 $p=0.0397$, protein/AdC7 10^9 > AdC7/AdC6 10^9 at wk 12 $p=0.0397$; TZBD PV: at wk 20 AdC7/protein 10^9 > protein/AdC7 10^9 $p=0.0055$, AdC7/protein 10^9 > AdC7/AdC6 10^9 $p=0.0055$.

Fig. 5. Induction of V2-specific Abs

(A) Ab binding to an immunodominant linear epitope within the V2 region of subtype C strain Du172. Sera were analyzed after the last immunization for each group; 1:100 dilution reported, mean $\pm$ SEM. Using pre-immunization values as baseline, both the AdC7/protein 10^{10} ($p < 0.0001$) and AdC7/AdC6 10^{10} ($p = 0.0255$) regimens elicited significant titers of V2-specific Abs (two-way ANOVA and Holm-Sidak multiple comparisons test). Statistical comparisons among groups were made by one-way ANOVA and Holm-Sidak multiple comparisons test. (B) Relative percentages of V2 Du172-specific Abs of the different isotypes at the week following last immunization using a serum dilution of 1:200. Overall the V2 Ab response trends toward a more pronounced frequency of IgM isotype as compared to the gp140-specific response. The symbols x/x over part B represent the significant differences between groups for the isotypes IgG2a and IgM, respectively (two-way ANOVA with Holm-Sidak multiple comparisons test). AdC7/protein 10^9 elicits a significantly greater frequency of IgG2a Abs than protein/AdC7 10^9 , $p < 0.0001$. At both 10^{10} and 10^9 , the percentage of IgG2a Abs elicited by AdC7/protein is greater than AdC7/AdC6,

$p=0.0204$ and $p=0.0062$, respectively. IgM Abs are elicited at a greater frequency by protein/AdC7 10^9 as compared to AdC7/protein 10^9 , $p=0.0345$.

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