**Combination recombinant simian or chimpanzee adenoviral vectors for vaccine
development**

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Abstract

Adenoviral vector-based vaccines are currently being developed for several infectious diseases and cancer therapy, but pre-existing seroprevalence to such vectors may prevent their use in broad human populations. In this study, we investigated the potential of low seroprevalence non-human primate rAd vectors to stimulate cellular and humoral responses using HIV Env glycoprotein (gp) as the representative antigen. Mice were immunized with novel simian or chimpanzee rAd (rSAV or rChAd) vectors encoding HIV gp or SIV gp by single immunization or in heterologous prime/boost combinations (DNA/rAd; rAd/rAd; rAd/NYVAC or Ad/rLCM), and adaptive immunity was assessed. Among the rSAV and rChAd tested, SAV16 or ChAd3 vector alone generated the most potent immune responses. The DNA/rSAV regimen also generated immune responses similar to the DNA/Ad5 regimen. ChAd63 prime/ChAd3 boost and ChAd3 prime/NYVAC boost induced similar or even higher levels of CD4⁺ and CD8⁺ T-cell and IgG responses as compared to Ad28/Ad5, one of the most potent combinations of human rAds. The optimized vaccine regimen stimulated improved cellular immune responses and neutralizing antibodies against HIV compared to the DNA/rAd5 regimen. Based on these results, this type of novel rAd vector and its prime/boost combination regimens represent promising candidates for vaccine development.

Keywords: adenoviral vectors, simian adenovirus, chimpanzee adenovirus, vaccine development, humoral immune response, cellular immune response

1. Introduction

Adenoviruses are non-enveloped icosahedral double-stranded DNA viruses that can broadly infect vertebrates including humans and non-human primates. There are 68 known serotypes of human adenoviruses divided into seven groups from A to G [1]. These adenoviruses have been studied for their pathogenicity and more recently as vectors for gene therapy and genetic vaccines. The large numbers of naturally existing adenoviruses from different hosts with varied tropism and immune stimulating capabilities represent a rich repertoire for the construction of recombinant vectors that have broad clinical applications.

Recombinant adenoviral vectors used as viral vectors for vaccines have many advantages and are being tested in clinical trials for HIV, malaria, and TB. Among these viral vectors, Ad5 is known to induce the strongest CD8⁺ T-cell responses compared to other serotypes and other viral vectors. However, the high prevalence of anti-Ad5 neutralization antibodies in human populations may restrict the use of rAd5, especially for optimizing CD8⁺ T-cell immunity. In previous human clinical trials, neutralizing antibodies against Ad5 correlate with reduced immunogenicity of the vector [2-5]. Therefore, alternative adenoviral vectors with low seroprevalence are critical for advancing these vaccines into broad clinical applications. Many human adenoviruses have been shown to either have high seroprevalence or to induce weak primary immune responses to transgenes [6]. Currently only rAd35 and rAd26-based vectors are in clinical evaluations for HIV vaccines. Non-human primate adenovirus-based vectors are attractive alternatives to human adenoviruses because these viruses do not circulate in human populations and are rarely neutralized by human sera. Several simian

adenoviruses have been also isolated from their hosts [7-10]. Those related to human adenoviruses are placed in the respective seven groups and the distantly related viruses are assigned to the distinct simian adenovirus A group. Based on phylogenetic analysis of genomic sequence, the chimpanzee adenoviruses ChAd3 and PanAd3 are classified in serotype C similar to Ad5, while ChAd63 and C68 are in serotype E. Recombinant ChAd3 vectors are reported to be similar to rAd5 since they induce similar levels of anti-tumor activity but have low seroprevalence in humans. Previous studies have focused on cellular immune responses generated with these vectors using gag as the antigen and neutralizing antibodies to HIV were not assessed [11]. Currently, rChAd3 and rChAd63 are being tested in clinical trials for the development of T-cell-based HCV, malaria, and Ebola vaccines [12, 13]. Application of these vectors to HIV vaccines, especially in prime/boost combination regimens using HIV Env as the antigen, has not been systematically investigated. Efficacious HIV vaccines are likely to include HIV Env as the immunogen. Indeed, recent studies in an SIV NHP challenge model suggest that Env immune responses correlate with and are required for vaccine efficacy [14, 15].

Combination of rAd with other genetic vectors including DNA, MVA/NYVAC and rLCMV in prime and boost regimen can further improve immune responses [12, 15]. Although these vectors can be used alone, specific regimens, for example, rAd/MVA or rAd/rAd and rAd/rLCMV are more potent [12, 14, 15].

In this study, we examined the ability of several novel simian and chimpanzee Ad vectors encoding HIV and SIV Env to generate cellular and humoral immune responses after single immunizations or in combination with DNA, NYVAC, or rLCMV. The

113 optimized regimen outperformed the DNA/rAd5 prime boost combination and represents
114 a candidate for further development.

115

2. Materials and methods

2.1. Animals

6-8 week-old female BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in the experimental animal facility of the Vaccine Research Center, NIAID, NIH (Bethesda, MD). All animal experiments were reviewed and approved by the Animal Care and Use Committee, VRC, NIAID, NIH and performed in accordance with all relevant federal and NIH guidelines and regulations.

2.2. DNA recombinant vaccines

The DNA plasmids expressing gp145 Δ CFI Δ V1V2 of HIV-1 clade B, gp145 Δ CFI of HIV-1 clade A, gp145 Δ CFI of HIV-1 clade C, or SIVmac239 gp140 were prepared by the methods described previously [16]. E1, E3, and E4-deleted rAd5 and E1-deleted simian and chimpanzee rAd were constructed and purified as described previously [10, 17, 18]. Simian adenoviruses SAV7, SAV11 and SAV16 were obtained from ATCC. The viruses were rescued and grown in an Ad5 E4 orf6-expressing HEK 293 cell line (293 orf6 cell line). Then, the E1 region of the viral genome was sequenced and deleted for the construction of recombinant vectors. The HIV or SIV Env gene under the control of a CMV promoter was inserted into the E1 region. Recombinant simian SAV and human rAd vectors were rescued in a 293 orf6 cell line. The chimpanzee rAd (rChAd3, rChAd63 and PanAd3) vectors containing Ad5 E4 orf6 in their E4 region as constructed previously were grown in HEK 293 cells [10]. Recombinant LCMV vectors were constructed as previously described [15]. NYVAC vectors were kindly provided by Dr. Tartaglia (Sanofi Pasteur, Allentown, PA) [19].

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140 ***2.3. Verification of Env transgene expression***

141 HEK 293 and A549 cells were transduced with rAd Env, 16 or 48 hours later,
142 respectively, the supernatants were collected and the cell-associated proteins were
143 extracted with cell lysis buffer (Cell Signaling Technology, Danvers, MA). Samples were
144 analyzed by SDS-PAGE and Western blotting using HIV-1 IIIB Strain Rabbit anti-gp120
145 Polyclonal (Advanced Biotechnology Inc., Columbia, MD) as a primary antibody and
146 ECL Rabbit IgG, HRP-Linked Whole Ab (GE Healthcare, Piscataway, NJ) as a
147 secondary antibody.

148

149 ***2.4. Immunization***

150 For single immunization, mice were immunized with rAd by intramuscular routes. For
151 DNA prime/rAd boost immunization, mice were intramuscularly primed with 15 µg of
152 DNA three times at two-week intervals and intramuscularly boosted with rAd two weeks
153 after the third DNA priming. For rAd prime/rAd intramuscular boost immunization, mice
154 were intramuscularly primed with 10^7 to 10^9 VP of rAd and intramuscularly boosted with
155 10^7 to 10^9 VP of rAd three to four weeks later. The immunized mice were sacrificed three
156 weeks after single immunization or two weeks after boost immunization. Five mice per
157 group were used in the studies unless indicated otherwise.

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159 ***2.5. Measurements of T-cell responses with Intracellular cytokine staining***

Single cells from the spleens of immunized mice were stimulated with an HIV Env peptide pool for 5 hours and the levels of effector cytokine-producing CD4⁺ and CD8⁺ T cells were examined by intracellular cytokine staining as described previously [20]. For measurement of IL-2-, IFN- γ - and TNF-producing CD4⁺ or CD8⁺ T cells, lymphocytes were plated with 2 $\times$ 10⁶ cells/well and incubated with or without HIV gp140B peptide pool for 5 hours in the presence of anti-CD28 (clone 37.51, BD Pharmingen, San Jose, CA), anti-CD49d (clone R1-2, BD Pharmingen) and 10 μ g/mL brefeldin A (Sigma). Peptides used in this study were 15 mers overlapping by 11 amino acids that spanned the complete sequence of the protein. Cells were sequentially stained with VIVID dye (Invitrogen, Eugene, OR), Fc block (BD Pharmingen) and mAbs against surface markers, such as PerCP-Cy5.5-conjugated anti-CD3 mAb (clone 145-2C11, BD Pharmingen), Alexa Fluor 700-conjugated anti-CD4 mAb (clone RM4-5, BD Pharmingen) and APC-Cy7-conjugated anti-CD8 α mAb (clone 53-6.7, Biolegend, San Diego, CA). The cells were permeabilized and fixed with Cytofix/Cytoperm (BD Pharmingen) and then stained with mAbs against cytokines, PE-conjugated anti-mouse IL-2 mAb (clone JES6-5H4), APC-conjugated anti-mouse IFN- γ mAb (clone XMG1.2) and PE-Cy7-conjugated anti-mouse TNF mAb (clone MP6-XT22, BD Pharmingen). The stained cells were examined by BD LSR-II (BD Pharmingen) and the data were analyzed by FlowJo software (Tree Star Inc., Ashland, OR).

2.6. ELISA for Env-specific antibodies

HIV or SIV Env-specific IgG were examined as follows: An ELISA plate with 96 wells was coated with 2 μ g/mL recombinant HIV or SIV Env protein at 4°C overnight and then

blocked with PBS plus 1% BSA at 37°C for an hour. 1:1000 diluted or serially diluted sera starting from 1:64 dilution from the immunized mice were added and incubated at 37°C for 2 hours. Horseradish peroxidase (HRP) conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was added and incubated at 37°C for an hour. 3,3',5',5-Tetramethylbenzidine (TMB, Sigma), HRP substrate was added to each well and the yellow color that developed after adding 0.5 M H₂SO₄ was measured at 450 nm.

2.7. Mouse IgG purification

Plasma IgG was isolated with rProtein G sepharose Fast Flow beads (GE Biosciences Inc.). Briefly, equal volumes of mouse serum in the same immunization group were pooled together and diluted into two volumes of PBS. The diluted serum was incubated with pre-washed and equilibrated rProtein G Fast Flow beads at 1:1 volume ratio for one hour at room temperature. The mixture was then loaded onto a column. Following a thorough wash, IgG was eluted with IgG elution buffer (Thermo Scientific) and neutralized immediately after elution. After dialysis the IgG solution was concentrated with an Amico Ultra centrifugal filter unit (Millipore) and the IgG concentration was determined using NanoDrop by reading OD₂₈₀ nm.

2.8. Viral neutralization assays

Neutralization was measured using single round infection by HIV-1 Env pseudoviruses and TZM-bl target cells as described previously [21]. Neutralization curves were fit by nonlinear regression using a 5-parameter hill slope equation. The 50% inhibitory

206 concentrations (IC50) were reported as the IgG concentrations required to inhibit
207 infection by 50%.

208

209 ***2.9. Data and statistical analysis***

210 Results are expressed as mean $\pm$ SE unless indicated otherwise. Statistical analyses were
211 performed upon comparisons made between the control groups and treated groups or
212 between treated groups using Student's t test.

213

3. Results

3.1. **Characterization** of recombinant simian and chimpanzee adenoviral vectors

expressing HIV or SIV Env

rAd vectors were purified and used to transduce human epithelial A549 cells (Fig. 1A) or HEK 293 cells (Fig. 1B) at different viral particle (vp) ratios per cell *in vitro* to confirm protein expression. Expression of Env protein was evaluated 16 or 48 hours after transduction by Western blotting. As ChAd vectors transduced A549 cells at low efficiency even after 48 hours, HEK 293 cells were used, and transgene expression was measured at an early time point (16 hr after transduction) to avoid viral replication. All the tested vectors expressed the Env transgene. Compared to rAd5, the expression levels were similar for simian rAd SAV7 and SAV11, and chimpanzee rAd ChAd3, while SAV16 expressed lower levels of Env (Fig. 1).

3.2. *Immunogenicity of simian or chimpanzee rAd following a single intramuscular injection*

To evaluate whether simian SAV and chimpanzee rAd vectors can induce immune responses comparable to rAd5, mice were immunized once intramuscularly with 10^7 to 10^{10} viral particles of each of the rAds. Three weeks later, Env-specific cellular and humoral immune responses were assessed (Fig. 2). All rAds induced Env-specific immune responses above background compared to the null vector (-). For cellular immune responses, cytokine IL-2 producing cells were not detectable and we used the percentage of IFN- γ^+ and TNF- α^+ in CD4 $^+$ and CD8 $^+$ T-cells as the measure of the potency with different vectors. The potency of the immune responses was dependent on

the dose and serotype of the rAd vector. There was a dosage dependent Env-specific CD8+ and IgG response from 10^7 to 10^8 VP dosage for all the vectors tested. The cellular and humoral immune responses stimulated by rAd5 or rSAV peaked around 10^9 to 10^{10} VP dosage, and rAd5-immunized mice developed stronger immune responses than any rSAV as measured by Env-specific CD4+, CD8+, and IgG responses (Fig. 2A, B). rSAV11 stimulated higher levels of humoral IgG but lower CD8+ T-cell responses than rSAV16. Alternatively, a single immunization with ChAd3 at a dose of 10^9 vp induced levels of intracellular IFN- γ^+ TNF- α^+ in CD4+ and CD8+ T-cells (Fig. 3A) and IgG (Fig. 3B) comparable to HIVgp140B delivered by rAd5. rChAd63 induced lower IgG responses, and Pan3 induced lower CD8+ and IgG responses than rAd5. All rAd vectors generated higher CD8+ than CD4+ T-cell responses.

3.3. Ability of rSAV to stimulate immune responses in combination regimens

Although immunization with rSAV alone stimulated lower immune responses than the rAd5 vector, we evaluated their potency as boost following DNA priming. Indeed the ability to boost a primed response may limit differences seen as single vaccines for priming. Mice were primed with DNA encoding HIV Env three times at 2-week intervals and boosted intramuscularly with rAd Env two weeks after the third DNA priming. Two weeks after the boost, the antibody and T-cell responses were assessed. Compared to DNA/rAd5 at 10^9 VP dosage, DNA/rSAV11 or DNA/rSAV16 stimulated comparable or increased immunity (Fig. 4A, B), although CD8+ responses were inferior at lower dosage (10^7 VP). Therefore, DNA/rSAV regimens at high dosages were

comparable to the DNA/rAd5 combination, suggesting that rSAV at appropriate doses may substitute for rAd5 as a boost for DNA.

Heterologous rAd in prime-boost regimens have been shown to stimulate more potent immune responses than DNA/rAd regimens [22]. Among them, the rAd serotype group D (rAd26, rAd28) prime and rAd5 boost regimen was the most potent in animal studies [22]. The rSAV/rAd5 regimen stimulated similar cellular (Fig. 5A) and humoral responses (Fig. 5B) compared to rAd28/rAd5. Therefore, rSAV was able to prime rAd5 to boost responses. However, SAV vectors were less effective as a boost, as rAd28/rSAV stimulated lower CD8⁺ and IgG responses than rAd28/rAd5 (Fig. 5C and 4D).

3.4. Combination regimens with rChAd

To develop a completely novel prime/boost regimen compared to rAds that are affected by global seroprevalence from prior exposure [11], we also compared rChAd as a prime or a boost vector in different combinations. Mice were boosted three weeks after priming, and immune responses assessed two weeks later. Heterologous rAd28 prime/rAd5 boost was used as a benchmark (Fig. 6A, B). In the heterologous rChAd/rChAd combinations, the rPan3/rChAd3 regimen stimulated higher CD8⁺ and similar CD4⁺ responses compared to the rAd28/rAd5 regimen. rChAd3/rChAd63, rChAd3/rPan3, rPan3/rChAd3 and rPan3/rChAd63 all induced IgG immune responses similar to rAd28/rAd5. We also tested these vectors in combination with other viral vectors including a NYVAC poxvectors and LCMV. NYVAC vectors are being used in clinical studies for an HIV vaccine and LCMV vectors have been shown to stimulate potent

immune responses in animal models [15]. Strikingly, the rChAd3/NYVAC regimen generated much higher CD4+, CD8+, and IgG immune responses than rAd28/rAd5. rPan3/NYVAC, and rChAd3 or rPan3/rLCMV also stimulated better (CD8+ and IgG) or similar (CD4+) immune responses compared to rAd28/rAd5. Therefore, we have identified specific combination regimens that outperformed the standard rAd28/rAd5 combination.

The potential of rChAd to stimulate anti-HIV IgG immune responses was further assessed using HIV EnvA and EnvC as the antigens. These are the same antigens used in the HIV vaccine clinical trial HVTN505. DNA/rChAd3 elicited similar levels of anti-HIV gp140A and gp140C binding IgG as DNA/rAd5 as measured by ELISA (Table 1, Fig 7). The heterologous prime/boost regimen rChAd3/rChAd63 stimulated a higher titer IgG response than DNA/rAd. Total IgG was purified from the sera of these immunized mice to assess HIV neutralizing antibodies, as the unpurified mouse sera contain non-specific activities in the assay. Only the heterologous rChAd3/rChAd63 regimen generated neutralizing IgG against two HIV strains tested, DJ263.8 (Clade A) and MW965.26 (Clade C). In particular, the regimen induced immune IgG that neutralized HIV strain MW965.26 at a low IC₅₀ of 1.4 µg IgG/ml.

4. Discussion

Adenoviruses from different serotypes may differ in their tropisms and in their interactions with the immune system that could affect the immune responses to a transgene product. We have previously tested several human adenoviral vectors, including Ad28 (or Ad26), Ad35, and Ad5 for their ability to induce HIV-specific immunity. rAd5 is the most potent for immunogenicity in animal models and seronegative humans, especially in stimulating CD8⁺ immune responses. Ad5 belongs to the human serotype C group, and its major cellular receptor is CAR. The highly immunogenic nature of Ad5 has been shown to relate to its ability to transduce broad antigen presenting cells including dendritic cells and monocytes, and to activate innate cytokine production [23, 24]. The major obstacles to the use of these human adenovirus vectors as vaccine carriers are the high prevalence of anti-vector neutralizing antibodies in human populations as in the case of rAd5 or lower immunogenicity to the antigen inserts in the case of rAd28 and rAd35.

Many strategies have been pursued to overcome pre-existing anti-Ad5 immunity [25, 26]. The capsid of rAd5 has been modified by the conjugation of immune-inert polymers including PEG and PLGA in order to shield the highly immunogenic epitopes on its surface from anti-Ad5 neutralizing Ab [27, 28]. As neutralizing Ab against Ad5 are generally made against the capsid or fiber [3, 29], these viral proteins of Ad5 can be swapped with the corresponding region of a different serotype of adenovirus [30, 31]. In addition, non-human Ads (ovine, porcine, bovine, simian and chimpanzee Ad) have been investigated as alternative vectors [6, 32]. Here, we tested the feasibility of using rare seroprevalent simian isolates from monkeys and chimpanzees as the vaccine vector. The

seroprevalence for chimpanzee and simian adenoviruses is relatively low [11]. Previous studies with rChAd3 were focused on cellular immune responses [10, 11]; here, we also measured humoral responses using Env as the antigen. We demonstrated that ChAd3 generated similar cellular and humoral responses to Ad5 after single immunization. Although the cellular receptors for rChAd3 are not known yet, it is likely to transduce and stimulate innate responses similarly to the other C serotype adenoviruses. It seems that vectors based on the serotype C group generated higher immune responses than other serotypes including serotype E and simian serotype A tested here.

For effective HIV vaccines, it is likely that prime-boost regimens will be required to generate long-term protective immunity as suggested in the RV144 clinical trials [33]. Therefore, we have examined the immunogenicity of these rAd vectors in various combination regimens with comparative dose-response for the development of an HIV vaccine. DNA prime/SAV boost and an SAV prime/Ad5 boost regimen elicited potent cellular and humoral immune responses. rSAV was as potent as rAd5 in boosting a DNA vaccine, and the DNA/rAd5 regimen has been shown previously to be more potent than DNA/rAd35 or rAd26 or rAd48 [6]. rAd5 boost stimulated higher CD8⁺ responses at 10⁷ vp dosage compared to higher dosage at 10⁹ vp. This is likely due to that rAd5 is potent and may have adverse effect at higher doses. The DNA/rAd boost regimen generated cellular responses with higher CD8⁺ than CD4⁺ responses, a trend similar to that observed in the rAd alone regimen, while a previous study with DNA/MVA has shown a more CD4⁺ T-cell-skewed response [34]. Therefore, specific vector combinations affect both the strength and quality of the immune responses.

rSAV failed to boost rAd28 immunization as efficiently as rAd5. On the other hand, rSAV was comparable to rAd28 as a prime for rAd5 vaccine. The mechanisms underlining the synergy of different combinations are still unclear. Combinations similar to rAd28/rAd5 and ChAd63/NYVAC can be found in the rAd26/rAd5 and ChAd63/MVA regimens tested previously [12, 22]. rAd26/rAd5 has been identified as the most potent among the rAd/rAd regimens in animal models and the ChAd63/MVA regimen has been highly immunogenic in human clinical studies [35, 36]. The potential of the rAd/rAd and rAd/recombinant pox vector is suggested by the results of these studies. Therefore, rAd can serve as the priming to rapidly induce immune responses and boosting with heterologous viral vectors can further enhance the potency of the vaccine.

DNA/rAd5 vaccine with the inserts used here did not induce detectable levels of neutralizing antibodies in mice. Although immunogenicity can be different in mice and humans, the low level of neutralizing activity shown here is consistent with the results obtained in the human clinical trials VRC009 and VRC010 using a similar DNA/rAd5 regimen [37]. In contrast, the rChAd3/rChAd63 regimen stimulated about 4-fold higher HIV gp140A and gp140C binding IgGs than DNA/rAd5. The immune IgG also neutralized two tier 1 HIV strains, generally considered easy to neutralize. As the small amount of purified IgG did not allow for elucidation of the mechanism of neutralization, the breadth of neutralizing activity remains to be further characterized. The Env sequence of MW963.26 and that of DJ263.8 shared 86% and 81% homology with the vaccine Env gp140C and gp140A, respectively. The V3 loop is the only highly conserved variable loop among the five variable loops from V1 to V5. Previous studies have demonstrated that the V3 loop is highly immunogenic, and anti-V3 neutralizing antibodies can

neutralize diverse strains of HIV-1 [38-41]. The disproportion of the ELISA titer and the neutralizing activity between the DNA/rAd group and the rChAd/rChAd group also suggests that the two vaccine regimens may stimulate qualitatively different IgG responses in terms of neutralizing antibodies. This result raised the possibility that vaccine regimen can influence the generation of neutralizing antibodies. The use of rAd can also reduce the number of priming compared to the use of DNA plasmid which is less potent. We conclude that specific combinations of chimpanzee adenoviral vectors may have the potential to induce significant cellular and humoral immunity and could be used more broadly in humans to avoid prior anti-vector immunity. The optimized prime/boost regimen could also be applied to diseases other than AIDS.

5. Conclusions

A vaccine must elicit the appropriate specificity, quality, and magnitude of immunity. Human clinical trials suggest that different vectors used for gene-based immunization may affect vaccine responses and hence their potential efficacy. This concern is relevant when immunity to the vaccine vector is seen, as with human adenovirus (rAd) 5. Here, we investigate the potential of low seroprevalence non-human primate rAd vectors to generate immunity against HIV. A specific recombinant chimpanzee rAd3 vector and related prime/boost combinations elicited potent immune responses. Such rAd vectors circumvent the problem of pre-existing immunity to human rAds used in current human trials. A qualitative difference in humoral immunity was elicited by these vectors compared to the regimen used previously in clinical trials. Specific priming with chimpanzee rAd3 vector and different boosting combinations may improve vaccine potency and efficacy.

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Author contributions

GJN designed and GJN, RMS, RAS and JRM supervised the study. CC, LW, SK, WK and SDS executed the study. JGDG, SC contributed to the construction of the rAd vectors. CC, LW, RAS and GJN wrote the paper. All authors have approved the final paper.

Conflict of interest statement

SC is an inventor on the patent application "Simian Adenovirus Nucleic Acid- and Amino Acid- Sequences, Vectors containing same, and uses thereof" (WO 2010/086189 A3). All mentioned patents are now under control of GlaxoSmithKline SA. The other authors declare no conflicts of interest.

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Regimen		Anti gp140A+C ELISA titer (GMT (95%CI))	IC ₅₀ (µg purified IgG/ml)	
Prime	Boost		DJ263.8 (clade A)	MW965.26 (clade C)
3xDNA HIVgp145A+C	Ad5 HIVgp140A+C	289,631 (42,884-1,956,000)	>200	>200
3xDNA HIVgp145A+C	ChAd3 HIVgp140A+C	121,775 (3,185-4,656,000)	>200	>200
ChAd3 HIVgp140A+C	ChAd63 HIVgp140A+C	1,081,000 (674,696-1,732,000)	45.0	1.4

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557 **Table 1. rChAd/rChAd combinations elicit potent anti-HIV immune IgG;**558 **comparison to DNA/rAd.** Four mice in each group were immunized with the indicated

559 vaccines. Two weeks after the last vaccination, sera were collected for ELISA analysis

560 and pooled to purify total IgG. Purified IgG was used in an HIV neutralization assay to

561 determine IC₅₀.

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Figure Legends

Figure 1. HIV gp140 immunogen encoded by the rAd vectors and the transduction of cells with simian and chimpanzee rAd. HIV gp160 and gp140 are shown with amino acid numbering based on the HXB2 sequence. The gp140 protein contains two deletions: one from residue 503 to 536, around the cleavage site (residues 510 and 511) and the fusion domain (residues 512 to 527); and the sequences between the two heptad repeats from residue 593 to 620 (A). A549 cells (B) or HEK293 cells (C) were transduced with the indicated rAd vector at 10, 100, and 1000 viral particles per cell (vppc) for 48 hours (A549 cells) or 16 hours (293 cells). The cell lysates were subjected to SDS-PAGE and Western blotting to determine HIV-1 gp140 or SIV gp140 expression.

Figure 2. T-cell and humoral responses induced by rSAV in mice compared to rAd5. Five BALB/c mice per group were intramuscularly immunized once with rAd5 null vector (-) at 10^8 vp or rAd HIV Env gp140B at the indicated dosages and euthanized 3 weeks later. (A) Single cells from the spleen were stimulated with the peptide pool of HIV Env for 5 hours, stained with mAbs against surface markers and cytokines, and then analyzed by flow cytometry. Data shows the percentages of IFN- γ and TNF-producing CD4⁺ or CD8⁺ T cells analyzed by intracellular cytokine staining. (B) HIV gp140B-specific IgG titers were measured in sera. Values are shown as mean $\pm$ SE; *, $p < 0.05$. 5: rAd5, s11: SAV11, s16: SAV16.

Figure 3. ChAd3 HIVgp140B single immunization with 10^9 vp generated comparable CD4+, CD8+ T-cell, and IgG responses to Ad5. Groups of five mice were immunized with Ad5 or ChAd3, ChAd63, PanAd3 HIVgp140B at 10^7 , 10^8 , and 10^9 vp intramuscularly. Spleens and serum were harvested 3 weeks after immunization to detect cellular immune responses by ICS and antibody response by ELISA. (A) % HIV Env specific intracellular IFN γ^+ TNF α^+ CD4+ T cells or % IFN γ^+ TNF α^+ CD8+ T cells. (B) Serum anti-HIV gp IgG antibody at 1:1000 dilution. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 4. Immunogenicity of prime boost combinations with DNA/SAV compared to DNA/Ad5. Groups of five BALB/c mice were intramuscularly immunized with DNA (D) three times at two-week intervals, boosted with rAd5 (5), SAV11 (s11) or SAV16 (s16) HIV gp140B Env, and then euthanized two weeks later. (A) Single cells from the spleen were stimulated with the peptide pool from HIV Env for 5 hours, stained with mAbs against surface markers and cytokines, and then analyzed by flow cytometry. Data shows the percentages of IFN γ and TNF-producing CD4+ or CD8+ T cells. (B) HIV gp140B-specific IgG titers were measured in sera. (C & D) Values are shown as mean $\pm$ SE; *, $p < 0.05$. D: DNA prime.

Figure 5. Immunogenicity of SAV/Ad5 or Ad28/SAV combinations compared to rAd28/rAd5. Groups of five BALB/c mice were intramuscularly primed and boosted with 1×10^8 VP of the indicated combination of adenoviral vectors encoding HIV gp140B Env at three week intervals and were euthanized two weeks later. (A) Single cells from

spleen were stimulated with the peptide pool from HIV Env for 5 hours, stained with mAbs against surface markers and intracellular cytokines, and then analyzed by flow cytometry. Data shows the percentages of IFN γ and TNF-positive CD4⁺ or CD8⁺ T cells. (B) HIV gp140B-specific IgG titers were measured in sera. Values are shown as mean $\pm$ SE; *, $p < 0.05$ vs. Ad28 / rAd5. 5: rAd5, s11: SAV11, s16: SAV1.

Figure 6. rChAd3 priming with NYVAC boosting immunization elicit potent CD4⁺ and CD8⁺ T-cell, and IgG, responses. Groups of five mice were primed and boosted with the indicated SIVmac239 gp140 encoding Ad vectors at 10^7 vp and NYVAC or rLCMV at 10^6 pfu intramuscularly at 4 week intervals. Spleens and serum were harvested 2 weeks after boost to detect cellular immune responses by ICS and antibody response by ELISA. (A) % IFN γ ⁺ TNF α ⁺ CD4⁺ T cells and % IFN γ ⁺ TNF α ⁺ CD8⁺ T cells. (B) Anti-SIV gp IgG antibody in serum. Ad28: 28; Ad5: 5; ChAd3: C3; ChAd63: 63; Pan3: P3; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 7. Elicitation of anti-HIV Env IgG and neutralizing antibodies in mice.

Groups of four mice were primed and boosted with the indicated regimen. Sera at week 2 post the last boost were collected and analyzed. (A) Anti HIV Env ELISA titer, geometric mean and 95% confidence interval was shown. (B) Neutralizing activities against two tier 1 HIV-1 viruses: DJ263.8 clade A and MW965.26 clade C. Purified IgG from the immunized mice were pooled together and used in the viral neutralization assays. Pre: prebled sera or IgG; D: DNA; 5: rAd5; C3: rChAd3 and C63: rChAd63.

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