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HIV-DNA Priming Alters T Cell Responses to HIV-Adenovirus Vaccine Even When Responses to DNA Are Undetectable


Many candidate HIV vaccines are designed to primarily elicit T cell responses. Although repeated immunization with the same vaccine boosts Ab responses, the benefit for T cell responses is ill defined. We compared two immunization regimens that include the same recombinant adenoviral serotype 5 (rAd5) boost. Repeated homologous rAd5 immunization fails to increase T cell responses, but increases gp140 Ab responses 10-fold. DNA prime, as compared with rAd5 prime, directs long-term memory CD8+ T cells toward a terminally differentiated effector memory phenotype with cytotoxic potential. Based on the kinetics of activated cells measured directly ex vivo, the DNA vaccination primes for both CD4+ and CD8+ T cells, despite the lack of detection of the latter until after the boost. These results suggest that heterologous prime-boost combinations have distinct immunological advantages over homologous prime-boosts and suggest that the effect of DNA on subsequent boosting may not be easily detectable directly after the DNA vaccination. The Journal of Immunology, 2011, 187: 3391–3401.

The goal of HIV vaccine development is to prevent or control HIV infection by inducing effective long-term immune memory. Vaccine-mediated induction of broadly neutralizing Abs has been unsuccessful due to extensive HIV envelope protein variability (1). Therefore, most vaccines in development primarily induce cellular immune responses.

Based upon experience with licensed vaccines, multiple vaccinations, known as a prime-boost regimen, will likely be needed for efficacy. Of interest is the effect on the magnitude and quality of long-term memory induced by priming and boosting with the same (homologous) or different (heterologous) vaccines. Heterologous vaccination could improve the quality of T cell responses (2).

To test this hypothesis, we designed a study to compare homologous priming and boosting with a recombinant adenoviral serotype 5 (rAd5) vaccine versus priming with DNA and boosting with rAd5 in healthy HIV-uninfected Ad5-seronegative adults [HIV Vaccine Trials Network (HVTN) protocol 068]. This is of particular interest considering the results of recent HIV vaccine efficacy trials. Repeated homologous boosting with rAd5 expressing Gag, Pol, and Nef was associated with increased rate of infection in some subgroups (3), whereas a recombinant canarypox vector prime expressing Env, Gag, and protease followed by recombinant gp120 protein boost was associated with a modest reduction in infection rates (4).

The vaccines used in this study each encode Env, Gag, and Pol. One treatment group received two rAd5 vaccinations (Ad5-Ad5), whereas the other group received two DNA primes and an rAd5 boost (DNA-Ad5). To evaluate the quality of the immune response, we assessed polyfunctionality by measuring cytokine production, evaluated cytotoxic potential, and quantified the types of memory cells elicited. We charted the temporal evolution of the response over multiple time points after each vaccination and at a longer-term time point 1 y after the first vaccination. DNA priming significantly affected the magnitude and character of the memory response to the rAd5 boost, even for CD8+ T cells that were not detectable following DNA.

Materials and Methods

Clinical trial

We conducted a phase 1 multicenter, double-blind, randomized, placebo-controlled study (http://www.clinicaltrials.gov; NCT00270218) comparing two different primes: rAd5 vector vaccine (group 1) or a DNA vaccine (group 2), each followed by an rAd5 boost. The DNA prime consisted of two injections 1 mo apart, rather than a single injection, because a prior study had shown that one dose rarely resulted in measurable T cell responses (5). This prior study included three doses of DNA, but we chose only to use two doses because the third dose minimally increased the response beyond the response following the second dose. The rAd5 prime consisted of a single injection because prior studies have demonstrated a high response rate following a single dose (6, 7). The 66 (33 in each group) participants were healthy HIV-1-uninfected adults (ages 18–50 y) lacking detectable pre-existing Ad5-neutralizing Abs (titers <1:12) (8).

The vaccines, developed by the National Institute of Allergy and Infectious Diseases, were created by the Vaccine Research Center at the National Institutes of Health and the National Institute of Allergy and Infectious Diseases. Each vaccine contained Env, Gag, and Pol (8). The vaccines used in this study each encode Env, Gag, and Pol.

This work was supported by the HIV Vaccine Trials Network and Statistical Center for HIV/AIDS Research & Prevention, a cooperative agreement with the National Institutes of Health Division of AIDS (National Institute of Allergy and Infectious Diseases) (U10 AI068614, U01 AI068618, and U01 AI068635). This work is also supported through the University of Washington Center for AIDS Research, a National Institutes of Health-funded program (P30 AI027757).

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The online version of this article contains supplemental material.

Abbreviations used in this article: GzB, granzyme B; HVTN, HIV Vaccine Trials Network; ICS, intracellular cytokine staining; PTE, potential T cell epitope; rAd5, recombinant adenoviral serotype 5; VRC, Vaccine Research Center.
Diseases Vaccine Research Center, encoded HIV-1 Env glycoprotein (clades A/B/C) and clade B Gag/Pol fusion gene; the four-plasmid DNA prime also encoded clade B Nef (included with Gag/Pol as the Gag/Pol/Nef fusion gene) (5, 6). Group 1 received 10^10 particle units rAd5 i.m. on day 0 and week 24; group 2 received 4 mg DNA vaccine i.m. via biojector on day 0 and week 4 and 10^10 particle units rAd5 i.m. at week 24. Placebo recipients (three in each group) received PBS for DNA and final formulation buffer for rAd5. Blood was collected by venipuncture on the day of each vaccination, weekly for the first 4 wk, and then 5 wk following the second DNA injection and each rAd5 injection. A final sample was obtained 365 d following the first injection.

The primary immunogenicity time points were protocol-defined as 4 wk following each rAd5 vaccination and 4 wk following the second DNA injection, which was chosen because prior trials rarely found measurable responses to one dose of the DNA vaccine (5). Most analyses used data from these prespecified time points, although they might not have been at the peak response for each individual.

The institutional review committee at each clinical site approved the protocol prior to study initiation, and participants completed a thorough informed consent process.

PBMC processing

PBMC were isolated and cryopreserved from heparin-anticoagulated whole blood within 8 h of venipuncture, as described previously (9). PBMC were thawed and, except for T cell activation analysis, cultured overnight at 37°C/5% CO_2 in RPMI 1640 [Gibco-BRL], containing 10% FCS [Gemini Bioproducts], 2 mM L-glutamine [Gibco-BRL], 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate) prior to stimulation.

Ex vivo stimulations

PBMC were assessed for ex vivo responses to pools of HIV-1 15-mer peptides (synthesized by Bio-Synthesis) overlapping by 11 aa covering global potential T cell epitopes (PTE) within Env, Gag, Pol, and Nef (10). All PTEs present at a frequency of ≥15% were identified and pooled by protein based on frequency from the Vaccine Virus Database. One pool (166 peptides) for each protein was used, which was sufficient for complete coverage of PTEs for Nef, but only 70, 87, and 71% of PTEs for Env, Gag, and Pol, respectively. The final concentration for each peptide was 1 μg/ml during stimulations. Staphylococcal enterotoxin B (Sigma-Aldrich) stimulation was the positive control, whereas peptide diluent (DMSO at a final concentration of 1%) was the negative control. The 6-h stimulation included brefeldin A (10 μg/ml) and IL-2 was lower for this assay versus the validated eight-color assay comparable between the assays, and data for TNF-α and IL-2 were used to calculate the percentage of PTE-specific T cells.

Flow cytometric assays

Eight-color intracellular cytokine staining assay. We used the validated eight-color intracellular cytokine staining (ICS) protocol as described previously (11). Reagents used in this and other panels are described briefly below and listed in Supplemental Table I. Cells were first stained with a combination of two antibodies (Dead Cell Stain and Live/Dead Fixable Dead Cell Stain). The 6-h stimulation included brefeldin A (10 μg/ml), and IL-2 expression was validated in bridging studies with the eight-color assay; for these cytokines, we report combined data from both assays. The TNF-α reagent was not comparable between the assays, and data for TNF-α are not included in the analyses presented in this paper.

Eleven-color ICSmemory marker assay. This assay was used to determine expression of four memory-defining markers (CCR7, CD45RA, CD27, and HLA-DR) and one pool of peptides. After incubation with dead cell stain, cells were surface-labeled with Ab reagents detecting the memory markers. Cells were fixed, permeabilized (11), and stained intracellularly with the remaining Ab reagents (Supplemental Table I). The frequency of cells producing IFN-γ and IL-2 was lower for this assay versus the validated eight-color assay (average of 20% lower, but variable). Therefore, the eight-color assay was performed on all samples as the primary endpoint assay, whereas this assay was performed as a secondary assay on selected samples.

Activation marker assay. Samples were stained after overnight culture of thawed PBMC. PBMC were stained with Aqua Live/Dead Fixable Dead Cell Stain (12) and then surface-stained with Ab reagents detecting CCR7, CCR5, CD27, CD38, and HLA-DR. Cells were fixed, permeabilized (11), and stained intracellularly with Ab reagents detecting CD3, CD4, CD8, Ki-67, and BeL-2.

Later experiments demonstrated that the frequency of activated cells was consistently higher when cells were examined immediately after thawing (median of 2.1 times higher, ranging to five times higher). However, we found no difference in the kinetics of the appearance and decline of activated cells whether cells were immediately examined or cultured overnight. Because most samples were examined the day after thawing, data previously presented in this study will not be recalculated.

Reagents. Dead cell stains were from Invitrogen/Molecular Probes. Abs were from BD Biosciences, except CD3–ECD (Beckman-Coulter), periforin (Tepnel/Diaclone), CD27 (eBioscience), and HLA-DR (BioLegend). We conjugated the periforin and CD57 Abs to Alexa 647 and Alexa 405 (Invitrogen), respectively.

Stained samples were collected from 96-well plates using the High Throughput Sample device (BD Biosciences), and 200,000–300,000 events from each sample were acquired on an LSRll flow cytometer capable of measuring 18 colors (BD Biosciences). All FACS analyses were performed using either FlowJo (Tree Star) or LabKey Flow (14). Positive responses and criteria for evaluable responses were determined as previously described (11), based on background measurements and the number of T cells examined. Because separate criteria are applied to CD4+ and CD8+ cells, the total numbers included in each ICS analysis can differ between the CD4+ and CD8+ T cell evaluations.

HIV Ab ELISA

Anti-Gag and anti-Env binding Ab responses were determined by validated ELISA as previously described (15, 16). Sera from cryopreserved samples were diluted and tested in duplicate in microtiter plates (Nunc) coated with purified p55 Gag (Protein Sciences), ConS gp410 (Dr. H-X. Liao, Duke University), and gp41 (Immunodiagnostics). Envelope proteins that match the envelopes encoded by the vaccine were not available, but we have found that consensus strain envelopes are extremely sensitive for detecting Ab responses to multiple strains when examining Ab responses in acute HIV infection (16). Plates were washed with an automated calibrated plate washer (Bio-Tek). OD was determined (M2 plate reader; Molecular Devices), and non–Ag-containing wells were subtracted from Ag-containing wells. Standard curves from the geometric mean of the plot of absorbance (450 nm) against the log of serum dilution, and sigmoidal curves were fit using a four-parameter logistic equation (Softmax Pro).

Quantitative Luminescent binding assay

Serum HIV-1–specific IgG responses to Clade B Consensus (BCon) gp120, group M Consensus (ConS) gp140, and Con6 gp120 (Dr. H.-X. Liao and B. Haynes, Duke University) were measured by a standardized HIV-1 Luminescent assay as previously described (16). Abs were measured as mean fluorescent intensities on a Bio-Plex instrument (Bio-Rad) under Good Clinical Laboratory Practice-compliant conditions. Positive standard curves in each assay included 2G12 mAb and purified IgG from HIV-positive sera that were fit using a four-parameter logistic curve fitting to calculate micrograms per milliliter equivalence concentration with the serum dilution that fit within the linear range of the curve (1:500 or 1:2500 dilution). Negative controls included HIV-negative human sera and blank beads.

Statistical analysis

Because few responses were detected to Nef and because the rAd5 vaccine did not encode Nef, no Nef data are included in this study. To reduce the adjustment for multiple comparisons and because fewer Gag and Pol responses were detectable with the DNA prime, statistical comparisons were performed only for the 4-wk and 6-mo time points postbooster on the overall response and Env-specific response.

ICS assay positivity was calculated based on comparisons between stimulated and negative control responses via a one-sided Fisher’s exact test. The resulting multiplicity-adjusted p values were used to determine positivity, with p < 1 × 10^-5 indicating a positive response. Comparisons of response magnitudes were performed using the nonparametric Wilcoxon rank sum tests and adjusted for multiple comparisons using Holm’s adjustment (17). Using the score method of Agresti and Coull (18), 95% confidence intervals for response rates were calculated.

Results

Randomization and safety

Participants in each group were well balanced for age, sex, and race (Supplemental Table II). All were Ad5 neutralizing Ab seronegative. As in earlier phase I clinical trials that evaluated the safety of
rAd5 alone, DNA alone, or DNA prime/rAd5 boost (5, 6), both regimens were well tolerated with minimal adverse reactions. Of the 30 participants randomized to receive each regimen, 26 Ad5-Ad5 participants and 28 DNA-Ad5 participants completed all vaccinations. All six control participants completed the regimen. Compared with control participants, more vaccinated individuals reported local and systemic reactogenicity, although severity levels were mainly mild (Supplemental Fig. 1).

**Immunogenicity as measured by T cell cytokine production**

To determine whether the vaccine induced T cell responses, we measured IFN-γ and/or IL-2 production by CD4+ or CD8+ T cells following stimulation with Env, Gag, or Pol peptide pools in an ICS assay. At each time point, the percentage of participants with a positive response was calculated (Fig. 1A, Table I). Comparisons were performed at the primary immunogenicity time points (4 wk after each rAd5 and 4 wk after the second DNA).

**Ad5-Ad5 group.** The first dose of rAd5 resulted in high CD4+ and CD8+ T cell response rates (63% at 4 wk after vaccination for each; Table I). Responses were relatively evenly distributed between Env, Gag, and Pol (61, 29, and 46%, respectively, for CD4+ T cells; 30, 42, and 50% for CD8+ T cells). Overall response rates were lower 4 wk after boosting with a second dose of rAd5 (39% for CD4+ and 57% for CD8+ T cells). Similarly, protein-specific response rates were decreased compared with after the prime, except for CD8+ Gag (Fig. 1A, Table I). The magnitude of responses as determined by percentage of cytokine-producing T cells was decreased for CD4+ T cells (overall median of 0.25% decreased to 0.14%), but was similar for CD8+ T cells (0.3%) following the boost as compared with the response to the prime (Fig. 1B, 1C).

**DNA-Ad5 group.** The DNA prime induced mainly CD4+ T cell responses (22% CD4+ versus 0% CD8+ responders at 4 wk after the second DNA; Fig. 1A, Table I), similar to the predominant CD4+ T cell response observed in prior DNA vaccine trials (5). DNA-induced responses were limited to Env. After the rAd5 boost, the percentage of responders increased for both CD4+ and CD8+ T cells (39 and 50%, respectively). Responses to Env predominated, but responses to Gag and Pol were also detected. Comparing the magnitude of Env-specific CD4+ T cell responses postprime and postboost, the median response within the first 3 wk postboost was increased (Fig. 1B), although at the primary immunogenicity time point (4 wk), the prime and boost responses were similar in magnitude (Fig. 1C).

**Comparison between treatment groups.** Following the prime, all response rates were higher for rAd5 than DNA. However, overall response rates were similar for the two treatment groups soon after the boost (4 wk) and at a longer-term time point (6 mo postboost) (Table I). Env-specific CD8+ T cell response rates trended higher for DNA-Ad5 at both the 4-wk and 6-mo time points postboost (22% for Ad5-Ad5 versus 45% for DNA-Ad5 at 4 wk and 4% versus 23%, respectively, at 6 mo; p = 0.2 for each). For Gag postboost, the CD8+ T cell response rate was comparable between DNA-primed and rAd5-primed subjects (25% versus 39%, respectively), whereas the CD4+ T cell response rate was low for both groups. For Pol postboost, the CD8+ T cell response rate was higher for the Ad5-Ad5 group (39% versus 10% for DNA-Ad5; p = 0.04); CD4+ T cell responses to Pol were low for both groups.

Comparing response magnitudes postboost, the CD4+ T cell responses overall and for Env were similar between treatment groups (Fig. 1C). However, Env-specific CD8+ T cell responses were significantly increased in the DNA-primed group compared with the rAd5-primed group at both the 4-wk and 6-mo time points postboost (median 0.23% versus 0.08% at 4 wk, and 0.18% versus 0.04% at 6 mo; adjusted p = 0.02 for each). These differences remained significant when absolute cell concentrations were compared (data not shown). Total CD8+ T cell response magnitudes were not significantly different, but we observed several responses above 1% only in the DNA-Ad5 group (Fig. 1C). Response magnitudes for other proteins were not compared due to the limited number of detectable responses in the DNA-Ad5 group.

Interestingly, except for Env-specific CD8+ T cell responses, a single dose of rAd5 administered as the prime resulted in higher response rates than a single dose of rAd5 administered after DNA priming (Table I, comparing 4 wk postprime for Ad5-Ad5 versus 4 wk postboost for DNA-Ad5). However, after adjusting for multiple comparisons, the difference was only significant for Pol-specific CD8+ T cell responses (50% for single rAd5 versus 10% for DNA/rAd5; adjusted p = 0.05).

**Cytokine profile**

Because polyfunctional responses are thought to be more effective at mounting successful immune responses (19–22), we examined cells coproducing IFN-γ and IL-2 (Fig. 2). Six months postboost, the DNA-Ad5 and Ad5-Ad5 groups differed in the proportion of dual-cytokine producing CD4+ T cells. Although not statistically significant when adjusted for multiple comparisons (adjusted p = 0.07), the large number of individuals in the DNA-Ad5 group with high percentages of these cells (>50%) was notable, suggesting a long-term effect of DNA priming.

**Memory marker expression**

To evaluate whether the vaccine regimens induce different types of memory T cells and to determine whether they change over time, we examined expression of two T cell differentiation markers (CD45RA and CD57) on vaccine-induced T cells. Naïve T cells express CD45RA, memory T cells lose CD45RA expression, and effector T cells re-express CD45RA and often coexpress CD57. We were unable to identify central and effector memory T cells due to technical problems with the CCR7 reagent typically used for this classification (23).

The majority of Env-specific CD4+ T cells (>80%) were CD45RA−CD57− (Fig. 3A). A small proportion were CD45RA+CD57+. These proportions were similar after priming and boosting with only a minor decrease in CD45RA+ cells postboost. No significant differences were seen between treatment groups.

In contrast, for Env-specific CD8+ T cells the CD45RA−CD57− and CD45RA+CD57− cell types were equally common 4 wk postprime. At later time points, postprime and postboost, the proportion of CD45RA−CD57− cells decreased and most cells expressed CD45RA, with some also coexpressing CD57 (Fig. 3A, Supplemental Fig. 2A). At 6 mo postboost, the DNA-Ad5 group had a greater percentage of CD45RA+CD57− Env-specific CD8+ T cells than the Ad5-Ad5 group (adjusted p = 0.1; Fig. 3A). The presence of the CD57−expressing CD8+ T cells is not simply a result of the rAd5 vaccine component of the DNA-Ad5 regimen, because few CD8+ T cells at 24 wk following a single dose of rAd5 express CD57 (Fig. 3A, second row). A similar trend was observed for Gag-specific T cells (Supplemental Fig. 2B), but statistical testing was not performed due to the fewer Gag- and Pol-specific T cells.

We previously demonstrated an association between bright staining for CD57, the purported marker of terminally differentiated cells (24), and perforin, allowing us to identify cells likely to have cytotoxic potential (25). We therefore examined expression of perforin, GzB, and CD57 for a few participants in each trial arm. Env-specific CD8+ T cells expressed intermediate, but not bright, levels of CD57 (Supplemental Fig. 2A). As expected, these cells did not express high levels of perforin. However, they
FIGURE 1.  Kinetics of percentage of responding individuals and percentage of cytokine-producing cells. For all graphs and all subsequent figures, the Ad5-Ad5 group is shown in red and the DNA-Ad5 group in blue. A. The percentage of individuals in each trial group with CD4+ or CD8+ T cell responses determined to be positive for Env, Gag, or Pol or for any protein (overall) is shown at each time point. B. The median percentage of cells producing IFN-γ and/or IL-2 in response to in vitro stimulation with Env, Gag, or Pol peptide pools, or the sum of these (total), is shown at each time point. Within each graph, only individuals with at least one positive response at any time point are included (this number is listed on each graph). C. The percentage of cells producing IFN-γ and/or IL-2 is shown for three time points: 4 wk after prime, boost, and 6 mo after boost. Within each graph, data are shown for all individuals with at least one positive response at any time point. The numbers on the graphs indicate adjusted p values (unadjusted in parentheses). For the DNA-Ad5 group, 4 wk postprime is 4 wk following the second DNA vaccination. The box plots indicate the median, 25th, and 75th percentiles; whiskers extend to the furthest point within 1.5 times the interquartile range.
For responders from the DNA-primed group, all peak CD4+ kinetics were similar, except that some individuals peaked after their peak response by 4 wk. The CD8+ rAd5-prime response peak CD4+ response by 2 wk after priming; all individuals reached 10% of individuals who had a prime response attained their postboost is charted over time in Fig. 4. For the rAd5-primed group, reaching their peak response to Env, Gag, and/or Pol postprime and ing each vaccination. The cumulative proportion of individuals response rates and magnitudes at multiple time points follow-

determine the kinetics of response decline postpeak, we measured To determine the time at which peak responses were attained and
expressed intermediate levels of GzB. Consistent with their higher frequency of CD8+ T cells expressing CD57, DNA-primed indi-

ters of these markers in combination (CD38 and HLA-DR

FIGURE 2. The percentages of Env-specific CD4+ and CD8+ T cells coproducing IFN-γ and IL-2. Only responses determined to be positive at the indicated time points are shown. The numbers on the graphs indicate adjusted p values (unadjusted in parentheses). For the DNA-Ad5 group, 4 wk postprime is 4 wk following the second DNA vaccination. The box plots indicate the median, 25th, and 75th percentiles; whiskers extend to the furthest point within 1.5 times the interquartile range.

The kinetics of peak responses postboost were more variable. Although >80% of individuals in either treatment group reached a peak CD4+ T cell response by 6 wk postboost, the time to peak stretched over the full 6 wk, unlike the more restrictive 2-wk period postprime. Postboost peak CD8+ T cell response kinetics were similar, except that more responses peaked beyond 6 wk postboost.

The duration of peak response and rate of decline can be ob-
served visually in Fig. 1A and 1B. The change in overall response rates and total magnitude suggests a two-phase decline, with a rapid decline shortly after the peak, resulting in a response level well above baseline that continued to slowly decrease.

Kinetics of activated T cells following vaccination

To assess induction of activated T cells in response to vaccina-
tion, we examined four markers of T cell activation (CD38, HLA-DR, Ki-67, and BcL-2) on CD4+ and CD8+ T cells. We first compared the frequency of T cells identified as activated by using pairs of these markers in combination (CD38 and HLA-DR versus Ki-67 and BcL-2). Although activated cells have been

Table I. Percentage of responders in each trial group postprime and postboost

<table>
<thead>
<tr>
<th>Prime</th>
<th>Boost</th>
<th>p Value^c</th>
<th>Ad5 Versus DNA-Ad5 p Value^c</th>
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<tbody>
<tr>
<td><strong>CD4+ T cells</strong></td>
<td><strong>CD4+ T cells</strong></td>
<td><strong>p Value^b</strong></td>
<td><strong>Ad5 Versus DNA-Ad5 p Value^b</strong></td>
</tr>
<tr>
<td>Env 14/23 (60.9); 4/18 (22.2); 41–78 9.0–45</td>
<td>8/23 (34.8); 7/18 (38.9); 19–55 20–61</td>
<td>5/27 (18.5); 5/25 (20); 8.2–37 8.9–39</td>
<td>0.05 shown, adjusted p value in parentheses.</td>
</tr>
<tr>
<td>Gag 7/24 (29.2); 0/18 (0); 15–49 0–18</td>
<td>3/23 (13); 0/18 (0); 4.5–32 0–18</td>
<td>1/28 (3.6); 0/25 (0); 0.6–18 0–13</td>
<td>0.01 (0.07)</td>
</tr>
<tr>
<td>Pol 11/24 (45.8); 0/18 (0); 28–65 0–18</td>
<td>5/23 (21.7); 2/18 (11.1); 9.7–42 3.1–33</td>
<td>2/28 (7.1); 1/25 (4); 2.0–23 0.7–20</td>
<td>0.02 (0.08)</td>
</tr>
<tr>
<td>Total 15/24 (62.5); 4/18 (22.2); 43–79 9.0–45</td>
<td>9/23 (39.1); 7/18 (38.9); 22–59 20–61</td>
<td>5/28 (17.9); 5/25 (20); 7.9–36 8.9–39</td>
<td></td>
</tr>
</tbody>
</table>

Data are number positive/total (percent positive); 95% confidence interval. Data were not available for all 30 vaccine recipients in each trial group. The major reason for missing data is a missing sample due to a missed study visit. Some data were filtered out due to assay protocol-defined criteria for high background response or low number of CD4+ or CD8+ T cells collected on the cytometer. No responses were detected in the six control participants, who are not shown in the table. Data for Nef are not shown in the table. One CD4+ and one CD8+ Nef response were detected in the DNA-Ad5 group at 24 wk postprime; one CD8+ Nef response was detected in the Ad5-Ad5 group postprime.

The percentages of Env-specific CD4+ and CD8+ T cells expressing CD57, DNA-primed individuals had higher percentages of GzB-expressing, Env-specific CD8+ T cells (Fig. 3B).
proposed to coexpress CD38 and HLA-DR (26), many activated cells we identified as Ki-67+BcL-2lo did not coexpress CD38 and HLA-DR. In the example shown in Fig. 5A, the percentage of Ki-67+BcL-2lo T cells that coexpressed CD38 and HLA-DR was 42% for CD4+ and 62% for CD8+ T cells. Because of the higher frequency of activated T cells detected and the more distinct staining pattern provided by Ki-67 and BcL-2, only they were used in subsequent analysis.

Unlike the kinetics for cytokine-producing cells, activated T cells had minimal variability in peak response timing, generally peaking by 2 wk postprime and 1 wk postboost (Fig. 5B). An increase in activated cells was commonly observed after rAd5, but rarely after DNA. For both treatment groups, the magnitude of the peak CD8+ T cell response for activated cells was generally greater than the peak response in detected cytokines (e.g., medians of ~0.3% by cytokine and 0.7% by activation for the Ad5-Ad5 group postboost). The frequency of activated cells rapidly declined following the peak, returning to prevaccination levels within 2 wk. Considerable variability in magnitude was observed (Fig. 5C), including some especially high CD8+ T cell responses (several above 2%, one at 7%, one at 13%).

**HIV-specific Ab responses**

Env- and Gag-specific Ab responses were measured by ELISA 4 wk postprime (first rAd5 or second DNA) and 4 wk postboost (Fig. 6A). The rAd5 prime induced a low-level Ab response...
significantly greater than baseline for all three Ags tested (adjusted \( p < 0.001 \)). DNA prime induced a low-level Ab response significantly greater than baseline for gp140 and p55 (adjusted \( p = 0.01 \) for each), but not for gp140. Unlike the T cell response, homologous immunization greatly enhanced postboost Env-specific Ab responses (~20-fold for gp140 and 8-fold for gp41). In contrast, the effect of the DNA prime on boosted Env-specific Ab responses was similar to that observed for T cell responses. Although the gp140-specific Ab response did not differ from baseline following DNA prime, DNA enhanced the subsequent rAd5-boosted response because this was significantly greater than the gp140-specific response to a single dose of rAd5 measured following the prime in the Ad5-Ad5 group (\( p < 0.001 \); Fig. 6A).

Comparing treatment regimens postboost, gp140-specific Ab responses were significantly higher in magnitude in the Ad5-Ad5 group compared with the DNA-Ad5 group (adjusted \( p = 0.02 \); Fig. 6A). Ab responses to gp140 and p55 were not significantly different, although there were some higher p55-specific responses in the Ad5-Ad5 group not observed in the DNA-Ad5 group. Concentrations of Env-specific Abs were also higher in the Ad5-Ad5 group when measured in a Luminex assay and expressed as equivalence concentration for 2G12 mAb (Fig. 6B). Neutralization of HIV strains represented in the vaccine constructs was not detected for either treatment group (data not shown).

**Discussion**

This study evaluated differences between homologous priming and boosting with an rAd5 vaccine and heterologous DNA prime/rAd5 boost vaccination. Homologous boosting greatly enhanced Env-specific Ab responses, but did not increase T cell responses. Despite limited postprime immune responses, DNA priming significantly enhanced the magnitude of postboost Env-specific Ab and CD4\(^+\) T cell responses and influenced the cytokine and memory marker profiles of the boosted T cell responses.

In addition to identifying vaccine-induced T cells based on Ag-specific ex vivo stimulation and cytokine production, we also directly identified activated T cells ex vivo. Recombinant Ad5, but not DNA, induced large percentages of activated CD4\(^+\) and CD8\(^+\) T cells shortly after immunization, and, consistent with a secondary immune response, the peak response was attained more quickly following the boost (1 wk) compared with the rAd5 prime (2 to 3 wk). Although few DNA-Ad5 individuals had detectable post-prime increases in activated cells, most had accelerated postboost kinetics similar to the Ad5-Ad5 group for both CD4\(^+\) and CD8\(^+\) T cells, with responses peaking by 1 wk, suggesting a secondary immune response. Thus, DNA is likely inducing undetected HIV-specific T cell responses. In our trial, this was especially notable for CD8\(^+\) T cells, because no participants had a detectable ICS response at the primary immunogenicity time point following DNA vaccination. Evaluation of immunogenicity and potential benefits of DNA vaccination should therefore not be based solely on ELISPOT or ICS assays measured directly after DNA vaccination, but should include these or alternate measures following boost.

Homologous priming and boosting is a common strategy for many licensed vaccines, which likely are effective due to induction of humoral immunity that benefits from repeated immunization with the same immunogen. However, the potential benefits of homologous immunization for T cell responses are not well defined. In our study, the magnitude of Ab responses to gp140 and gp41 were increased significantly after rAd5 boost; conversely, a second vaccination with rAd5 did not enhance T cell responses, potentially due to antivector immunity induced by the first vaccination. However, the divergent effects on cellular and humoral immune responses indicate a more complex mechanism than simply impairing expression of the HIV proteins from the rAd5 boost. Indeed, Ag presentation may be affected by Ad5-specific neutralizing Abs forming immune complexes with the vector, resulting in an effect on dendritic cell maturation (27).

In contrast to homologous rAd5 vaccination, rAd5 boosting of DNA resulted in an increased response rate and magnitude compared with the response detected after the DNA prime, most notably for Env-specific CD8\(^+\) T cells. At 6 mo postboost, the proportion of Env-specific CD8\(^+\) T cells expressing CD57 and GzB also tended to be higher in the DNA-Ad5 group. CD57 expression is associated with terminally differentiated T cells with high cytotoxic effector potential, which are armed for a quick response to pathogens (24, 25, 28). Induction of these cells by a vaccine is likely beneficial to control viral replication at its earliest stage, but the cells must be maintained over time. Although CD57 is proposed to be a marker of replicative senescence...
Activated T cells detected following vaccination. 

A. Higher proportion of activated T cells is detected using Ki-67 and Bcl-2 as compared with CD38 and HLA-DR. Flow cytometric staining profile in one Ad5-Ad5 individual 1 wk after the rAd5 boost shows expression of Ki-67 and Bcl-2 in the left panels. Numbers indicate percentage of CD4+ or CD8+ T cells that are Ki-67+Bcl-2lo. Right panels show expression of CD38 and HLA-DR. The Ki-67+Bcl-2lo cells are shown in red as overlay on the total T cells (gray). Numbers indicate the percentage of the Ki-67+Bcl-2lo cells that coexpress CD38 and HLA-DR.

B. The median percentages of CD4+ and CD8+ T cells that are Ki-67+Bcl-2lo at each time point are shown for the Ad5-Ad5 (upper panels) and DNA-Ad5 (lower panels) groups. Medians are for a total of 16 vaccine recipients in each trial group for whom the assay was performed.

C. The percentage of CD4+ and CD8+ T cells that are Ki-67+Bcl-2lo at 2 wk postprime (first rAd5 or second DNA) and 1 wk postboost. The box plots indicate the median, 25th, and 75th percentiles; whiskers extend to the furthest point within 1.5 times the interquartile range.
induced CD8+ T cells were detected 6 mo postboost and thus have a long-term memory. The majority of CD8+ T cells induced by vaccinia rather than memory, T cells (23), but our data and other studies indicate they can still contribute to long-term memory. The majority of CD8+ T cells induced by vaccinia and yellow fever vaccines, known to produce long-lived protection, highly express CD45RA and persist and maintain proliferative ability for many years (24), these cells are capable of rapid expansion (29). We also detect these cells at 6 mo postboost, indicating their potential for long-term maintenance.

The CD8+ T cells from both treatment groups had similar CD45RA expression, with nearly all postboost Env-specific cells expressing CD45RA. Nonnaive CD45RA+ T cells have been classified as effector, rather than memory, T cells (23), but our data and other studies indicate they can still contribute to long-term memory. The majority of CD8+ T cells induced by vaccinia and yellow fever vaccines, known to produce long-lived protection, highly express CD45RA and persist and maintain proliferative ability for many years (22, 30). In our trial, vaccine-induced CD8+ T cells were detected 6 mo postboost and thus may similarly contribute to long-term memory.

The magnitude of the CD4+ T cell response at 4 wk and 6 mo postboost was similar for both treatment groups, but the cytokine profile of Env-specific CD4+ T cells differed at the 6-mo time point. A subset of DNA-Ad5 individuals had high percentages (>50%) of Env-specific T cells that coproduced IFN-γ and IL-2. This might be beneficial because polyfunctionality is linked to mounting a more effective host response (19–22). Thus, DNA priming can favorably influence the long-term vaccine-induced cytokine profile, at least in some individuals.

Except for Env-specific CD8+ T cell responses, a single dose of rAd5 resulted in the highest response rates not only when compared with a second rAd5 dose, but also when compared with rAd5 administered after DNA prime. The dampening of rAd5-induced responses to some HIV proteins following DNA vaccination is possibly due to a DNA-induced suppressive effect. This effect may not be due to DNA as the method of vaccination, but rather due to the design of the DNA-encoded immunogens. The three Env proteins (clades A, B, and C) are encoded on different plasmids, each including a secretory sequence. Gag, Pol, and Nef are encoded on one plasmid. The National Institutes of Health Vaccine Research Center has recently developed a new DNA vaccine that encodes Gag, Pol, and Nef on separate plasmids (31); trials with this DNA vaccine also include a third DNA vaccination. Those trials do not demonstrate a lower postboost Gag-specific CD4+ T cell response rate like we observed (32), suggesting that these changes may have alleviated any potential suppressive effect for Gag, although not for Pol. Finally, our trial was restricted to Ad5-seronegative participants. In contrast to potential suppression, DNA priming of rAd5 is reported to mitigate a potential decreased response to rAd5 vaccination in Ad5-seropositive individuals (32).

The Step Study, a prior efficacy trial of an rAd5-vectorized vaccine, failed to reduce HIV infection or reduce HIV viral load (3, 13). Instead, early analyses revealed an increased incidence of HIV infection in Ad5-seropositive vaccinees. Concern was raised as to whether the vaccine increased the number of activated CCR5+ T cells, thus increasing the availability of target cells susceptible to infection. In our study (restricted to Ad5-seronegative participants), we found an increase in activated T cells, and many activated CD4+ T cells expressed CCR5 (data not shown). However, this increase was short-lived, at least as measured in blood, suggesting only a brief period of increased risk, unless vaccine-induced activated T cells persist at mucosal sites. Although kinetics indicate that the majority of activated cells are likely to be HIV-specific, some may be vector-specific. If so, differences in the design of the rAd5 vectors used in the two trials may affect vector persistence and thus persistence of activated cells. In particular, our VRC rAd5 vaccine has additional Ad5 gene deletions that may decrease vector persistence. Supporting this, a recent study demonstrated that the VRC rAd5 vaccine did not induce expansion of vector-specific CD4+ T cells or increase the activation state of Ad5-specific CD4+ T cells (33).
Our data demonstrate no T cell benefit for repetitive boosting with the same viral vector vaccine. Instead, a single dose of rAd5 induced the highest response rates and magnitudes for many HIV proteins, regardless of DNA priming. However, DNA priming altered the character of the postboost T cell response, even without inducing a detectable T cell response itself. This was most notable for Env, which, as noted above, has better expression than the other proteins in the DNA vaccine used in this trial (5). The newer DNA vaccine may extend the benefits of DNA priming to the other proteins. It should also be noted that there are other methods for improving the immunogenicity of DNA vaccines. One is through coadministration of DNA that encodes for cytokines that can potentially serve as adjuvants (34, 35). Another is through administration by electroporation. Both of these methods may further enhance the priming potential for DNA vaccination (36, 37).

Unlike the T cell response, homologous boosting with the same viral vector dramatically increased Env-specific Ab responses. These Abs bind to Env but do not neutralize HIV. The recent RV144 trial suggests potential benefits to inducing nonneutralizing Abs, as nearly all vaccinees developed Env-specific binding Abs with minimal neutralizing activity (4). This trial also highlights the potential utility of adding recombinant protein as one component of a vaccine regimen. The protective effect in RV144 was likely Ab-mediated because vaccination was associated with decreased HIV acquisition but not viral control in those who became infected. Our study, considered in the context of these other trials, suggests that vaccine regimens may need to include both heterologous vaccine modalities to optimize T cell responses and homologous boosting to increase Ab responses.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figures and Tables

### Supplemental Table I. Flow cytometric staining panels.

<table>
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<tr>
<th>Laser</th>
<th>Channel</th>
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<th>10-Color</th>
<th>11-Color</th>
<th>Activation</th>
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<tr>
<td>Violet</td>
<td>V450&lt;sup&gt;1&lt;/sup&gt;</td>
<td>ViViD&lt;sup&gt;1&lt;/sup&gt;</td>
<td>CD57* (Alx 405) AViD&lt;sup&gt;2&lt;/sup&gt;</td>
<td>CD57 (Alx 405) AViD&lt;sup&gt;2&lt;/sup&gt;</td>
<td>HLA-DR (Pacific Blue) AViD&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>V525&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Blue</td>
<td>FITC</td>
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<td>CD4*</td>
<td>CD4*</td>
<td>Ki-67*</td>
</tr>
<tr>
<td></td>
<td>PerCP-Cy5.5</td>
<td>CD8*</td>
<td>TNF-α*</td>
<td>CD8*</td>
<td>CD8*</td>
</tr>
<tr>
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<td>IL-2*</td>
<td>IL-2*</td>
<td>Bcl-2*</td>
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<td>CD3*</td>
<td>CD4*</td>
<td>CD45RA</td>
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<td>IFN-γ*</td>
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<td>Red</td>
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<td>Perforin* (Alx647)</td>
<td>Perforin* (Alx647)</td>
<td>IFN-γ* (APC)</td>
<td>CD38 (APC)</td>
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<td>Alexa 700</td>
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<td>TNF-α*</td>
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<td></td>
<td>R780&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td>CD4* (APC-H7)</td>
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</tbody>
</table>

<sup>1</sup>LIVE/DEAD Fixable Violet Dead Cell Stain (Violet Viability Dye)
<sup>2</sup>LIVE/DEAD Fixable Aqua Dead Cell Stain (Aqua Viability Dye)
<sup>3</sup>In these channels, different fluorescent dyes are used in different panels. Dyes are shown under the marker in the lists of reagents for each panel.
*Denotes intracellular reagent.
Supplemental Table II. Demographic Data.

<table>
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<th>Ad5-Ad5</th>
<th>DNA-Ad5</th>
<th>Control</th>
</tr>
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<tbody>
<tr>
<td>n</td>
<td>30</td>
<td>30</td>
<td>6*</td>
</tr>
<tr>
<td>Age (years)</td>
<td>28 (19-50)</td>
<td>28 (18-50)</td>
<td>35 (32-40)</td>
</tr>
<tr>
<td>Gender (%male)</td>
<td>16 (53%)</td>
<td>17 (57%)</td>
<td>3 (50%)</td>
</tr>
<tr>
<td>Race</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>White</td>
<td>27 (90%)</td>
<td>22 (73%)</td>
<td>4 (67%)</td>
</tr>
<tr>
<td>Black</td>
<td>3 (10%)</td>
<td>4 (13%)</td>
<td>0</td>
</tr>
<tr>
<td>Hispanic</td>
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<td>3 (10%)</td>
<td>1 (17%)</td>
</tr>
<tr>
<td>Asian</td>
<td>0</td>
<td>1 (3%)</td>
<td>0</td>
</tr>
<tr>
<td>Multiracial</td>
<td>0</td>
<td>0</td>
<td>1 (17%)</td>
</tr>
<tr>
<td>Vaccination frequency</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0 (rAd5, 1st DNA)</td>
<td>30 (100%)</td>
<td>30 (100%)</td>
<td>6 (100%)</td>
</tr>
<tr>
<td>Day 28 (2nd DNA)</td>
<td>NA</td>
<td>30 (100%)</td>
<td>3 (50%)</td>
</tr>
<tr>
<td>Day 168 (rAd5 boost)</td>
<td>26 (87%)</td>
<td>28 (93%)</td>
<td>6 (100%)</td>
</tr>
</tbody>
</table>

Data are number (%) or median (range)

*As a control for the Ad5-Ad5 group, three individuals received adenoviral vector final formulation buffer (FFB) at Day 0 and Day 168; as a control for the DNA-Ad5 group, three individuals received PBS at Day 0 and Day 28, and FFB at Day 168.
Supplemental Figure 1. Local and systemic reactogenicity. Shown are the percentages of participants in each study group reporting reactogenicity within 72 hours of each study vaccination. The following symptoms are considered as local reactogenicity: pain, tenderness, erythema and induration at the injection site. The following symptoms are considered as systemic reactogenicity: malaise and/or fatigue, myalgia, headache, nausea, vomiting, chills, arthralgia and above normal temperature. Within each category of local and systemic reactogenicity, the maximum level of severity for any symptom is plotted as mild, moderate or severe.
Supplemental Figure 2. Expression of CD45RA and CD57 on vaccine-induced CD4+ and CD8+ T cells. A. A representative flow cytometric staining profile for Env-specific
CD4+ and CD8+ T cells is shown for one individual from the DNA-Ad5 treatment group six months post-boost. Env-specific T cells were identified by expression of IFN-γ and/or IL-2 and are shown as red dots overlaying the overall CD4+ or CD8+ T cells (gray contours). Expression of CD45RA and CD57 are shown in the upper panels. For the CD8+ T cells, granzyme B, perforin and CD57 are shown in the lower panels. The Env-specific CD4+ T cells were negative for these markers and are not shown.  

B. As for Env-specific CD8+ T cells, Gag-specific CD8+ T cells show increased expression of CD57 in the DNA-Ad5 group (blue dots) compared with the Ad5-Ad5 group (red dots). The percentage of IFN-γ and/or IL-2-producing CD4+ or CD8+ T cells expressing each combination of CD45RA and CD57 in response to stimulation with Gag, Pol or SEB is shown at six months post-boost.