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


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**Supplementary Material**

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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 I 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 a single injection but increases the response beyond the response following the second dose. The rAd5 prime consisted of a single injection but 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, support through the University of Washington Center for AIDS Research, a National Institutes of Health Division of AIDS (U01 AI068614, U01 AI068618, and U01 AI068635). This work is supported by the National Institute of Allergy and Infectious Diseases (U01 AI068641, U01 AI068618, and U01 AI068653). This work is also supported through the University of Washington Center for AIDS Research, a National Institutes of Health-funded program (P30 AI027757). Address correspondence and reprint requests to Dr. Stephen C. De Rosa, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue North, LE-200, Seattle WA 98109. E-mail address: sderosa@fhcrc.org

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.

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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) (U01 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).
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 a Gag/Pol/Nef fusion gene) (5, 6). Group 1 received 10^10 particles unit 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 particles unit 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 6 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 this DNA vaccine (5). Most analyses were done 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 thoroughly 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% CO₂ 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 assayed 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) (PTE) within Env, Gag, Pol, and Nef (10). All PTEs present within each pool were included in the assay, 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 anti-IL-2 (anti-IL-2) (each at 1 µg/ml, BD Biosciences).

**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 Live/Dead Fixable Dead Cell Stain, then fixed, permeabilized, and stained intracellularly with fluorescent-labeled Ab reagents detecting CD3, CD4, CD8, IFN-γ, IL-2, TNF-α, and IL-4. For some assays, anti–IL-4 was replaced with anti-perforin that was conjugated to Alexa 488.

**Ten-color ICS assay.** The 10-color ICS assay (13) included an evaluation of granzyme B (GzB) and CD57 expression using the same reagents as the modified 8-color assay (including perforin) except for TNF-α–FITC, CD4–allophycocyanin-H7, GzB–Alexa 700, CD57–Alexa 405, and Aqua Live/Dead Fixable Dead Cell Stain. IFN-γ 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 ICS/flow cytometry assay.** This assay was used to determine expression of four memory-defining markers (CCR7, CD45RA, CD27, and HLA-DR) and CD4–allophycocyanin-H7, GzB–Alexa 700, CD57–Alexa 405, and Aqua Live/Dead Fixable Dead Cell Stain. IFN-γ 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.

**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 presented in this study is day 0 or week 4 responses. 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 polyclonally functional 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.
Kinetics of vaccine-induced T cell responses

To determine the time at which peak responses were attained and determine the kinetics of response decline postpeak, we measured response rates and magnitudes at multiple time points following each vaccination. The cumulative proportion of individuals reaching their peak response to Env, Gag, and/or Pol postprime and postboost is charted over time in Fig. 4. For the rAd5-primed group, the majority of peak responses occurring between 2 and 4 wk after that dose.

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 observed 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 vaccination, 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 Group</th>
<th>4 Wk*</th>
<th>Boost</th>
<th>6 Mo*</th>
</tr>
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<tbody>
<tr>
<td>Ad5-Ad5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+ T cells</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Env</td>
<td>14/23 (60.9); 4/18 (22.2); 41–78</td>
<td>8/23 (34.8); 7/18 (38.9); 19–55</td>
<td>5/27 (18.5); 5/25 (20); 8.2–37</td>
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<tr>
<td>Gag</td>
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<td>3/23 (13); 0/18 (0); 4.5–32</td>
<td>1/28 (3.6); 0/25 (0); 0.6–18</td>
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<tr>
<td>Pol</td>
<td>11/24 (45.8); 0/18 (0); 28–65</td>
<td>5/23 (21.7); 2/18 (11.1); 9.7–42</td>
<td>2/28 (7.1); 1/25 (4); 2.0–23</td>
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<tr>
<td>Total</td>
<td>15/24 (62.5); 4/18 (22.2); 43–79</td>
<td>9/23 (39.1); 7/18 (38.9); 22–59</td>
<td>5/28 (17.9); 5/25 (20); 7.9–36</td>
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<tr>
<td>CD8+ T cells</td>
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<tr>
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<td>7/23 (30.4); 0/18 (0); 16–51</td>
<td>5/23 (21.7); 9/20 (45); 9.7–42</td>
<td>1/26 (3.8); 6/26 (23.1); 0.2</td>
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<tr>
<td>Gag</td>
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<td>3/28 (10.7); 6/26 (23.1); 0.7–19</td>
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<tr>
<td>Pol</td>
<td>12/24 (50); 0/19 (0); 31–69</td>
<td>9/23 (39.1); 2/20 (10); 22–59</td>
<td>8/28 (28.6); 1/26 (3.8); 0.04</td>
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<td>Total</td>
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<td>13/23 (56.5); 10/20 (50); 37–79</td>
<td>11/28 (39.3); 10/26 (38.5); 24–58</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 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 observed 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.
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

FIGURE 3. Expression of CD45RA and CD57 on Env-specific CD4+ and CD8+ T cells. A. The four potential combinations of CD45RA and CD57 expression are shown on the x-axis. The percentage of Env-specific CD4+ or CD8+ T cells (identified by expression of IFN-γ and/or IL-2) expressing each combination is shown on the y-axis at each time point. B. The percentage of Env-specific CD8+ T cells expressing Granzyme B at 6 mo postboost is shown. For all graphs, 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.
FIGURE 4. Representation of the time at which peak response is reached. Cumulative distribution graphs are shown for the time to reach the peak total response after the prime (left panels) or the boost (right panels) for CD4+ (upper panels) and CD8+ (lower panels) T cells. Time (weeks) is shown on the x-axis, and the proportion of individuals reaching the peak on or before that time is shown on the y-axis. Peak response is determined as the maximum within an individual for the CD4+ or CD8+ T cells producing IFN-γ and/or IL-2 summed for Env, Gag, and Pol. Only individuals with at least one positive response after prime or after boost are shown. Some individuals reached the peak response >10 wk following the boost.

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 gp14 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 postprime 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
FIGURE 5. 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 likely to long-term maintenance. 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, 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 Ad5 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-vectored 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).
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DISCLOSURES

The authors have no financial conflicts of interest.

REFERENCES


