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Polytopic fractional delivery of an HIV vaccine alters cellular responses and results in increased epitope breadth in a phase 1 randomized trial

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Summary

Background Elicitation of broad immune responses is understood to be required for an efficacious preventative HIV vaccine. This Phase 1 randomized controlled trial evaluated whether administration of vaccine antigens separated at multiple injection sites vs combined, fractional delivery at multiple sites affected T-cell breadth compared to standard, single site vaccination.

Methods We randomized 90 participants to receive recombinant adenovirus 5 (rAd5) vector with HIV inserts *gag*, *pol* and *env* via three different strategies. The Standard group received vaccine at a single anatomic site (n = 30) compared to two polytopic (multisite) vaccination groups: Separated (n = 30), where antigens were separately administered to four anatomical sites, and Fractioned (n = 30), where fractions of each vaccine component were combined and administered at four sites. All groups received the same total dose of vaccine.

Findings CD8 T-cell response rates and magnitudes were significantly higher in the Fractioned group than Standard for several antigen pools tested. CD4 T-cell response magnitudes to Pol were higher in the Separated than Standard group. T-cell epitope mapping demonstrated greatest breadth in the Fractioned group (median 8.0 vs 2.5 for Standard, Wilcoxon p = 0.03; not significant after multiplicity adjustment for co-primary endpoints). IgG binding antibody response rates to Env were higher in the Standard and Fractioned groups vs Separated group.

Interpretation This study shows that the number of anatomic sites for which a vaccine is delivered and distribution of its antigenic components influences immune responses in humans.

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Keywords: HIV; Fractionated delivery; Polytopic vaccination; Ad5; Epitope breadth

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Research in context

Evidence before this study
There is a critical need to develop a safe and effective HIV vaccine. While vaccines targeted at generating robust T-cell responses have failed to prevent acquisition, the breadth of responses did correlate with viral clearance. In addition, immunodominance to a limited number of epitopes can reduce the potential of eliciting broad and clinically relevant T-cell responses. To address this issue, we designed the present study to determine if polytopic vaccine delivery would affect the breadth of T-cell responses compared to a standard, single site injection.

Added value of this study
This Phase 1 study demonstrated the feasibility of vaccinating participants polytopically at multiple sites. Ninety participants were enrolled and received HIV gene inserts either in the left deltoid, separated so each gene was injected into a different limb, or fractioned so all four genes were injected into four limbs. The fractionated strategy induced more robust T-cell responses as well as higher epitope breadth than the standard delivery.

Implications of all the available evidence
Our data demonstrate that a fractioned antigen delivery can achieve higher epitope breadth than standard vaccination and has implications for vaccine delivery strategies going forward. Multi-limb vaccination may be a way of increasing B or T-cell responses, especially epitope breadth.

Introduction

Despite decades of research and clinical trial testing, a vaccine to prevent HIV acquisition remains elusive. Potential correlates of risk and protection were identified in the RV144 trial, the only efficacy trial to show modest efficacy, which include both humoral and cellular responses.[1,2] Over the past 15 years, four other efficacy trials failed to show clinical efficacy in reducing HIV acquisition despite similar immunogenicity as RV144. Subsequent studies suggest that an effective HIV vaccine may benefit from elicitation of both T-cell and antibody responses.[3,4]

Preclinical data and studies including people living with HIV have shown clear evidence that HIV/simian immunodeficiency virus (SIV)-specific CD8+ T-cell responses, including number of epitopes recognized (i.e., breadth), are associated with control of viral replication and protection against disease progression.[5–9] Despite this, the HVTN 505 and Step trials, which involved vaccine regimens that induced cytotoxic T-cell (CTL) responses, failed to offer protection from infection or modification of disease.[10,11] The elicitation of responses to epitopes that do not play a role in viral clearance or protection from infection as well as mutational escape from initial CTL responses[12–14] are possible explanations for the lack of efficacy of CTL-based vaccines.

Immunodominance, which occurs when only limited antigenic responses are elicited despite numerous potential epitopes,[15] can be a problem if the immunodominant epitopes are irrelevant to viral control.[16,17] In the setting of HIV's high antigenic diversity and variation, a focused T-cell response may result in preferential targeting of irrelevant epitopes or antigenic competition, leading to ineffective memory responses upon challenge. This immunologic focusing is observed during HIV infection, where a limited number of epitopes are targeted during untreated early and chronic infection.[18–20] Similarly narrow responses were observed by epitope mapping of an adenovirus 5 (Ad5)-vectored vaccine encoding *gag-pol* (subtype B) and *env* (subtypes A, B and C) in HVTN 054, where a median of three CTL epitopes were targeted.[21] Vaccination with the same recombinant Ad5-*gag-pol* regimen with and without Env protein demonstrated greater CD8+ T-cell epitope breadth against Gag and Pol in the absence of Env, suggesting antigenic competition.[22] In the Step efficacy trial, CD8+ T-cell responses to ≥3 Gag epitopes were associated with improved viral clearance.[23]

Preclinical studies of candidate HIV vaccines in NHP and other animal models, where relatively broad T-cell responses and some degree of protection from disease progression has been seen,[24–27] frequently employ multisite (or polytopic) vaccination strategies because of practical issues relating to a substantial injection volume of vaccine going into a relatively small animal or in a deliberate attempt to avoid antigenic competition.[27–29] Comparison of polytopic and monotopic Dengue vaccination in NHP showed that while single-site vaccination resulted in more rapid cytokine production, durability was higher in NHP vaccinated via polytopic, separated antigen delivery.[30] Murine studies have also demonstrated the ability to overcome immunodominance by separating vaccine antigens across several anatomic sites.[31–34] It was recently shown that co-administration of DNA and protein HIV vaccines as fractional polytopic delivery in NHP elicited higher immune responses and offered greater protection from SIV than separating the DNA and protein to contralateral sites.[35] Several HIV vaccine studies have administered vaccines into the deltoids of both arms and showed high T-cell responses to Gag and Pol antigens.[36,37] Thus, there is potential of polytopic delivery

vaccination to broaden immune responses to vaccination in humans.

The development of an HIV vaccine with protective efficacy in humans may depend in part on the ability to stimulate broad adaptive cellular immune responses capable of significantly curtailing viral replication in the host. In this Phase 1 randomized controlled trial, we examined the whether the different strategies affected immunodominance and thus shaping of the CTL responses, and evaluated the value of polytopic vaccination strategies and distributing antigen at different anatomic sites in broadening vaccine responses to multiple epitopes. We administered the same immunogens at the same total dose at a single timepoint via three different strategies: 1) Standard, where rAd5-*gag-pol/env A/B/C* was injected intramuscularly (IM) in the upper right arm (the standard method of vaccination); 2) Separated, where rAd5-*gag-pol* was given IM in the right arm, rAd5-*env A* in the left arm, rAd5-*env B* in the right thigh, and rAd5-*env C* in the left thigh; and 3) Fractioned, where rAd5-*gag-pol/env A/B/C* was given IM at 1/4 the dose at each of the four anatomical sites. We hypothesized that fractional vaccine administration would enhance cellular immune responses compared to single site vaccination.

Methods

Experimental study design

HVTN 085 was a randomized double-blind Phase 1 trial conducted at nine clinical research sites in Chicago, IL (1), Boston, MA (1), New York City, NY (3), Philadelphia, PA (1), San Francisco, CA (1), Rochester, NY (1), and Nashville, TN (1) (ClinicalTrials.gov identifier: NCT01479296). Participants were healthy adults living without HIV-1 and adenovirus 5 (Ad5) seronegative between 18 and 50 years of age screened for eligibility (full eligibility criteria listed in Protocol) and subsequently provided written informed consent. Participants were randomized 1:1:1 to receive 1 mL intramuscularly (IM) of one of three treatment regimens. The Standard group received VRC rAd5-*gag-pol/envA/B/C* injected at 1×10^{10} particle units (PU) in the right arm and final formulation buffer (FFB) in the left arm, right thigh, and left thigh. The Separated group received 0.5×10^{10} PU VRC rAd5-*gag/pol* injected in the right arm and 0.17×10^{10} PU rAd5 *envA*, rAd5 *envB* and rAd5 *envC* in the left arm, right thigh and left thigh, respectively. The Fractioned group received VRC rAd5-*gag-pol/envA/B/C* injected at 0.25×10^{10} PU in the right arm, left arm, right thigh, and left thigh. The study was conducted over 7 months with annual participant health contacts for five years following initial study injection. The full protocol is available as Supplementary Material.

Randomization sequences, made by computer-generated random numbers, were provided to each site through a web-based randomization system. Randomization was done in 10 blocks of size 9 to ensure balance across groups. At each site, the pharmacist with primary responsibility for dispensing study products was charged with maintaining security of the treatment assignments. Allocation concealment was performed.

Products and administration

VRC-HIVADV014-00-VP (VRC rAd5 vaccine; rAd5 *gag-pol/env A/B/C*) is a replication-deficient, combination vaccine containing a mixture of four recombinant Ad5 vectors, each expressing one of the four HIV-1 antigens gp140 (subtype A, EnvA), gp140 (subtype B, EnvB), gp140 (subtype C, EnvC), and Gag-Pol (subtype B) driven by the cytomegalovirus immediate-early promoter. Manufacturing was based upon production in a proprietary cell line (293-ORF6), yielding replication deficient Ad vectors.

VRC-HIVADV038-00-VP (rAd5 *envA*), VRC-HIVADV052-00-VP (rAd5 *envB*), VRC-HIVADV053-00-VP (rAd5 *envC*), and VRC-HIVADV054-00-VP (rAd5 *gag-pol*) are single-component rAd5 vaccines comprised of single Ad5 vectors individually expressing the antigens listed previously.

VRC-DILUENT013-DIL-VP is the full name of FFB used in the Control group. All products were formulated as sterile liquid injectables for IM injection. All products were supplied by the Vaccine Research Centre, NIAID. The primary outcome assessors were masked with respect to the study treatments.

Outcomes

The primary outcomes of this study were to determine whether separation of HIV antigenic components of VRC rAd5 HIV vaccine across multiple injection sites increases the breadth and magnitude of T-cell responses. Responses were measured four weeks post vaccination and assayed by both intracellular cytokine staining and ELIspot. Secondary outcomes included whether administration of the separate antigenic components of VRC rAd5 HIV vaccine across multiple injection sites or division of a dose of rAd5 across multiple injection sites increases the response rate to vaccination.

Safety and reactogenicity evaluation

Safety evaluations included physical examinations, standard clinical chemistry, haematological tests and urinalysis. Local and systemic reactogenicity were assessed for up to 7 days following the vaccination or until resolution. Adverse events (AE) were recorded for 7 months of participation in addition to annual contacts for 5 years to collect information on important medical events including HIV-1 infection, pregnancies, new chronic conditions, and serious AEs. AEs were graded for each participant until completion of follow-up according to the Division of AIDS Table for Grading the

Severity of Adult and Paediatric Adverse Events, Version 1.0, 2004 (Clarification August 2009). Participants were tested for HIV-1 at study screening and 4 and 7 months follow up. Trial safety of participants was overseen by the HVTN 085 Protocol Safety Review Team and HVTN Safety Monitoring Board.

Binding antibody multiplex assay (BAMA)

HIV-1 specific IgG binding antibody (bAb) responses (1/50 dilution) against 8 HIV-1 antigens (Supplementary Table S1) were measured using a validated binding antibody multiplex assay (BAMA) on a Bio-Plex instrument (Bio-Rad) as previously described.[38] IgA bAb responses were also measured (Supplementary Table S1). The Bio-Plex readout is background-subtracted mean fluorescent intensity (MFI), where background refers to a blank well on a plate, and a concentration based on a standard curve. The positive control was purified polyclonal IgG from persons with HIV and negative controls were intravenous immunoglobulin and blank beads.

Intracellular cytokine staining (ICS)

Flow cytometry for CD4+ and CD8+ T-cell responses were measured using a validated ICS assay as described elsewhere.[39] Peripheral blood mononuclear cells (PBMC) were collected from participants at baseline and 28 days after vaccination and cryopreserved from whole blood as previously described.[40] The PBMC were stimulated with synthetic peptide pools (Supplementary Table S1). The negative control was unstimulated cells. As a positive control, cells were stimulated with phytohemagglutinin. There were no replicates except for the negative control, which had two replicates. Antibodies used in the study are listed in Supplementary Table S2. All antibody staining reagents have been obtained from commercial vendors who provide certificates of analysis. We titrate all these reagents prior to use.

ELISpot assay

Ex vivo HIV-specific T-cell responses were assessed with a validated IFN-γ ELISpot assay using cryopreserved PBMC stimulated overnight with synthetic peptides. Vaccine matched HIV-1 peptides representing EnvA, EnvB, EnvC, GagB and PolB were used for this study. Peptides were validated using the MabTech/Millipore assay. In addition to 3 experimental wells for each peptide, 6 negative control wells and 2 positive control wells (containing phytohemagglutinin) were tested for each specimen. Overall, 100,000 PBMC were used per well with spot-forming cells (SFC) per million cells as the readout. The cryopreserved PBMC were first stimulated with master-pools of ~50–150 15mers and mini-pools of ~12 15mers. For mini-pools eliciting positive responses, additional PBMC were tested for each 15mer contained within the mini-pool. Lab personnel were blinded to treatment group assignment.

T-cell epitope mapping by ELISpot

IFN-γ ELISpot assays were performed to map the epitopes targeted by the HIV-specific T cells and to assess the relative magnitude of the responses using 15-mer peptides overlapping by 11 aa matching the Env, Gag and Pol vaccine immunogen sequences. The sequences used were Env (subtype A), Env (subtype b), Env (subtype C), Gag (subtype B) and Pol (subtype B) (Supplemental Figure S1). All peptides were received as individual peptides, reconstituted, pooled and validated in both the ELISpot and ICS assays and were used at a final concentration of 1 μg/mL per peptide (Bio-Synthesis, Lewisville, TX). Epitope mapping was conducted in stages. Mini-pools were tested for participants with a significant response to protein-level pools. Positive responders to each mini-pool were investigated for response to the individual peptide constituents of the positive mini-pool.

Statistics

ICS

Positivity of ICS responses of individual cytokines or cytokine combination was ascertained by a one-sided Fisher's exact test, as described previously (Horton et al.).[41] Primary endpoints indicated comparisons of Standard vs Separated and Standard vs Fractioned. Fisher's exact tests were used to compare differences in response rates between groups. The differences in response rates and their corresponding 95% confidence intervals were estimated using Barnard's unconditional exact test implemented with version 3.2 of the exact package in R. Wilcoxon rank sum tests were used to compare differences in the magnitudes of responses among positive responders.

IFN-γ ELISpot epitope mapping

MIMOSA (Mixture Models for Single-Cell Assays) was used to determine a positive response to a specific 15mer peptide.[42] The MIMOSA test is used to compare cell counts between antigen-stimulated and unstimulated samples from a subject to identify significant differences. Cell counts are modelled by a binomial distribution and information is shared across subjects by means of a prior distribution placed on the proportion parameter of the binomial likelihood.

After obtaining response calls via MIMOSA, the number of epitopes was assessed. In general, each positive response was counted as one epitope. However, if two positive 15mers for a given participant overlapped by at least eight amino acids with two or fewer mismatches, this was counted as one epitope. Participants without 15mer data were those without positive responses at the mini-pool level and were thus considered to have zero epitopes. Boxplots show the number of epitopes for each participant (breadth) by treatment group. The mid-line of the box denotes the median and

the ends of the box denote the 25th and 75th percentiles. Whiskers extend to the minimum and maximum. RCDF (reverse cumulative distribution function) curves are used to show the distribution of the number of epitopes (breadth) by treatment group. Each point on the RCDF curve displays the proportion of subjects with breadth ≥x for a given breadth x noted on the x-axis. Whiskers extend to the most extreme data points that are no more than 1.5 times the interquartile range.

Wilcoxon rank sum test was used to test the difference of T-cell breadth by treatment groups. All p-values are two sided.

BAMA

Samples from post-enrolment visits were declared to have positive responses if they meet three conditions: (1) MFI–blank values ≥Antigen Specific cut-off (based on the average + 3 standard deviations of 60 seronegative plasma samples), (2) MFI–blank values > 3 times the baseline (day 0) MFI–blank values, and (3) MFI values are >3 times the baseline MFI values. Background adjusted MFI and concentrations were used to summarize the magnitude at a given time-point. Plots include data from responders only (coloured circles) with box plots superimposed on the distributions. The mid-line of the box denotes the median and the ends of the box denote the 25th and 75th percentiles. The whiskers that extend from the top and bottom of the box extend to the most extreme data points that are no more than 1.5 times the interquartile range (i.e., height of the box) or if no value meets this criterion, to the data extremes. Fisher's exact tests were used to compare differences in response rates between groups. Wilcoxon rank sum tests were used to compare differences in the magnitudes of responses among positive responders. No multiple comparison adjustment was applied.

Sample size calculations

The main goals of this trial regarding immunogenicity outcomes involve comparisons of the breadth of response and comparisons of magnitude of response between vaccine groups (Group 1 [Standard] vs Group 2 [Separated] and Group 1 vs Group 3 [Fractioned]). Breadth of response is measured by the number of epitopes targeted by cellular immune responses elicited by each vaccine. The test for comparisons of breadth responses between groups will be a test of superiority. Comparisons of magnitude of response between vaccine groups will be a test for non-inferiority. Responses (magnitude and breadth) are considered co-primary endpoints and the Type I error of 0.05 is shared equally among these comparisons. Thus, for each of the 2 comparisons between groups and for each of the co-primary endpoints (4 tests in total) the nominal size (Type 1 error) of tests will be .05/4 = 0.0125.

The power for formal comparisons of the mean number of epitopes between vaccine groups is shown in the table below. These calculations were done based on comparison of mean response within a Poisson regression model assuming a 10% loss of immunogenicity data. These calculations assume the mean number of epitopes is 2, which is based on the epitope mapping of vaccine recipients who received one 1×10^{10} PU dose of the rAd5 vaccine encoding clade B gag-pol and env A/B/C in HVTN 054. A Type 1 error rate of 0.0125 is used. For example, if the true mean number of epitopes is 2, then the minimum mean number of epitope responses in order to detect a difference with 90% power is 3.7.

Power for comparison of the mean number of epitopes between vaccine groups (n1 = 27, n2 = 27)

True difference mean number of epitopes	Power to detect a difference in mean number of epitopes
2.0 vs 3.0	45.3
2.0 vs 3.2	62.0
2.0 vs 3.4	75.9
2.0 vs 3.5	81.9
2.0 vs 3.7	89.5
2.0 vs 4.0	96.6

The table below shows the power to detect non-inferiority for several log10 magnitude differences. The test uses all available data and assumes a 10% loss to follow-up. Thus, the power calculations are based on an effective sample size of 27. These calculations assume the log10 transformed magnitudes are normally distributed with standard deviation of 0.316 for both groups and a mean of 2.87 for Group 1. These values of standard deviation and mean were based on ELISpot vaccine recipient data in the 1×10^{10} PU dose group of HVTN 054. A t-test with Type 1 error rate of 0.0125 was used to test the null hypothesis of 0%, 10%, and 20% difference from the true mean. The non-inferiority margin was set to 40% reduction in the mean magnitude log10 (0.4 × 744). For example, there is 81% power to detect a magnitude of 20% reduction in spot-forming cells (SFC)/10^6. If there is truly a 20% reduction in the difference, then there is an 81% chance of declaring non-inferiority with a margin of log10 (0.4 × 744).

Power for comparison of magnitude between vaccine groups (n1 = 27, n2 = 27)

True difference in magnitude Log10 SFC/10^6 (GMT SFC/10^6)	Power to detect non-inferiority
0%	97%
10%	93%
20%	81%

The table below shows the overall power of magnitude and breadth combined. These calculations assume magnitude and breadth are independent. For example, there is 83% overall power to detect a true difference in mean number of epitopes of 2.0 vs 3.7 with a 10% true difference in magnitude.

Overall power of magnitude and breadth

True difference mean number of epitopes	True difference in magnitude Log10 SFC/10^6 (GMT SFC/ 10^6)		
	0%	10%	20%
2.0 vs 3.5	79%	76%	66%
2.0 vs 3.7	86%	83%	72%
2.0 vs 4.0	93%	90%	78%

Multiple comparison adjustment for co-primary endpoints.

There are four co-primary endpoints for comparisons of the Separated and Fractionated groups to the Standard group for breadth of response as measured by ELISpot and magnitude of response as measured by the percent of CD4+ and CD8+ T cells expressing IFN-γ and/or IL-2 to any antigen as measured by ICS. Thus, for each of the breadth co-primary endpoints (2 of 4 tests in total) the nominal size of tests will be .05/ 4 = 0.0125. To account for dual ICS magnitude endpoints (CD4+ and CD8+) endpoints, the nominal size will be further adjusted as 0.0125/2 = 0.00625. All reported p-values are unadjusted with significance reported as 0.05 with the exception of the co-primary endpoints for breadth with significance reported for p-values below 0.0125 and magnitude comparisons below 0.00625.

Ethics

The study was approved by local IRBs of the clinical research sites (Chicago, University of Illinois at Chicago IRB #2011-0918; Columbia, Fred Hutch IRB #7645D; New York Blood Center, Fred Hutch IRB #7645C; Brigham, Partners Human Research Committee #2011P002458/PHS; Nashville, Vanderbilt IRB #111445; Philadelphia, Fred Hutch IRB #7645B; Rochester, University of Rochester IRB #RSRB00039790; San Francisco, Fred Hutch IRB #7645A) (ClinicalTrials.gov identifier: NCT01479296) and the FDA. Written informed consent in English was obtained from all participants.

Role of funders

The funders had no role in study design, data collection, data analyses, or interpretation.

Results

Study population and schema

Ninety participants were randomized and vaccinated between February 8 and September 24, 2012. Thirty participants were allocated to each vaccine group (Standard, Separated or Fractioned). Overall, 85 of 90 participants (94%) completed follow-up (Fig. 1). The majority were assigned male sex at birth (62%) and non-Hispanic White (74%), with an overall median age of 26 years (range 18–50) (Supplementary Table S3).

Safety

Consistent with previous reports, the vaccinations were generally well tolerated,[21,22,43,44] without differences between groups (Supplementary Figure S2, Supplementary Table S4). Distributing antigen to multiple sites, up to four, did not affect tolerability or safety and thus the non-standard delivery strategies of this regimen had a similar safety profile as standard vaccination.

Antigen-specific T-cell responses elicited by single site and polytopic vaccination

CD4+ and CD8+ T-cell response rates and magnitudes to peptide pools covering individual vaccine antigens (Env A, Env B, Env C, Gag, and Pol subdivided into two pools of Pol 1 and Pol 2) as well as Any antigen, Any Env and Any Pol were measured by intracellular cytokine staining (ICS) at 4 weeks post vaccination. There were no statistically significant differences in CD4+ T-cell response rates between the Standard group and either of the experimental groups, although overall the rates tended to be higher for Env than Gag and Pol antigens (e.g., Gag: Separated vs Standard, 34.5% vs 43.3%, Fisher's exact test p = 0.596, response rate difference −8.9%, 95% CI −33.6%, 16.8%; Fractionated vs Standard, 39.3% vs 43.3%, Fisher's exact test p = 0.795, response rate difference −4.0%, 95% CI −29.5%, 21.5%) (Fig. 2, Supplementary Table S5). The percent of CD4+ T cells expressing IFN-γ and/or IL-2 was similar between groups for all antigens tested except Pol 2, where the Separated group median was higher than Standard (0.175% vs 0.069%, Wilcoxon p = 0.03) (Fig. 2).

CD8+ T-cell response rates to Env C were significantly lower in the Standard group than Separated (36.7% vs 65.5%, Fisher's exact test p = 0.04, response rate difference 28.9%, 95% CI 2.8%, 51.5%) (Fig. 3, Supplementary Table S6). Standard group rates were also significantly lower than Fractioned to Env A (40% vs 67.9%, Fisher's exact test p = 0.04, response rate difference 27.9%, 95% CI 1.7%–51.1%), Pol 1 (24.1% vs 65.4%, Fisher's exact test p = 0.003, response rate difference 41.2%, 95% CI 14.1%–62.1%), and Any Pol (44.8% vs 80.8%, Fisher's exact test p = 0.01, response rate difference 35.9%, 95% CI 10.1%–57.5%). Participants in the Standard group had lower CD8+ T-cell

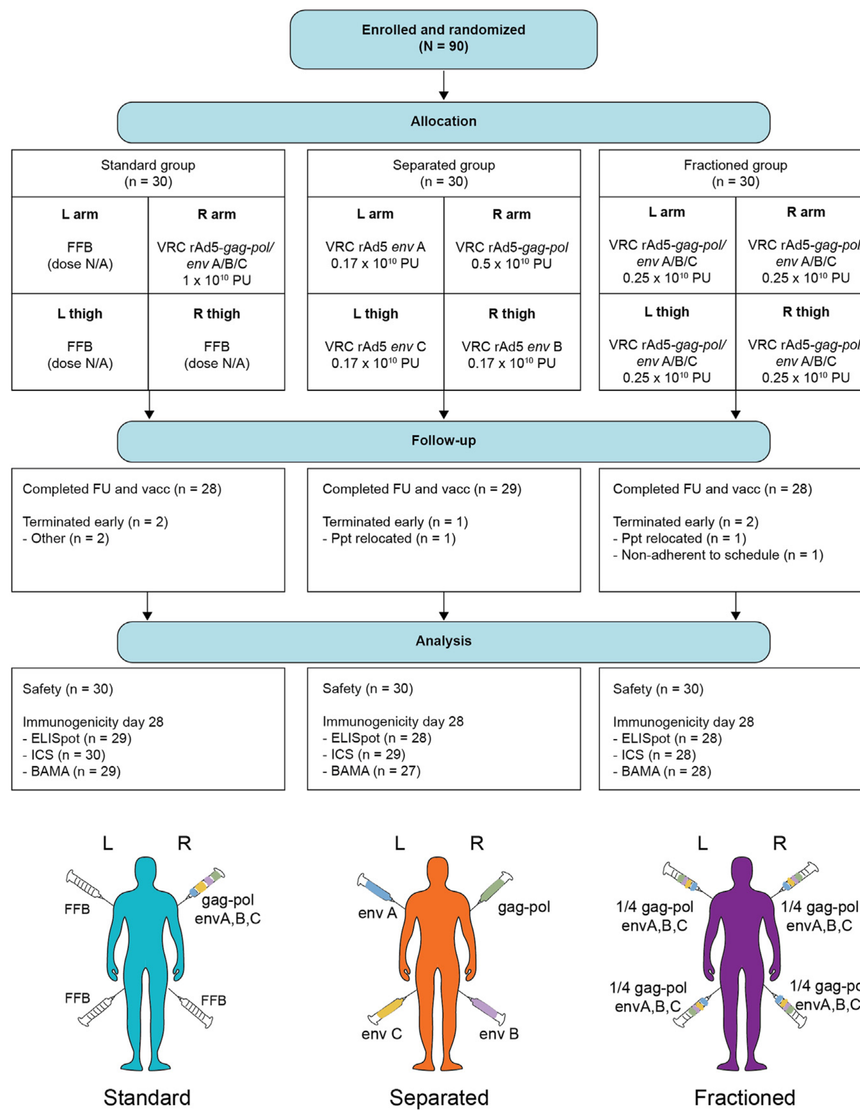

Fig. 1: **CONSORT diagram and trial description**. L = left; R = right; FFB = final formulation buffer, PU = plaque units; FU = follow-up; ICS = intracellular cytokine staining; BAMA = binding antibody multiplex assay.

response magnitudes than Fractioned to Env B (0.137% vs 0.372%, Wilcoxon p = 0.02), Any Env (0.167% vs 0.381%, Wilcoxon p = 0.02), and Any antigen (0.377% vs 1.259%, Wilcoxon p = 0.001; significant after multiplicity adjustment for co-primary endpoints).

In summary, the polytopic groups tended to have higher CD8+ T-cell response rates than the Standard, with the Fractioned group significantly higher or tending to be higher for all antigens tested except Gag. Differences for CD4+ T cells were more limited, with significance only for CD4+ T-cell magnitudes higher in the Separated than Standard group for Pol responses.

Fractional delivery vaccination leads to increased T-cell breadth

In order to determine if polytopic vaccination increased the breadth of T-cell responses, we compared the number of T-cell epitope responses elicited for each participant by IFN-γ ELISpot at 4 weeks post vaccination. The total number of targeted epitopes to all five vaccine inserts in the Fractioned group was significantly higher than Standard (median 8.0 vs 2.5, Wilcoxon p = 0.03), with no statistically significant difference between Standard and Separated groups (2.5 vs 2.0, Wilcoxon p = 0.84) (Fig. 4a). A similar trend was observed for all Env (across all three vaccine inserts 4.0 vs 1.0, Wilcoxon p = 0.02) (Fig. 4b) and additionally for epitopes

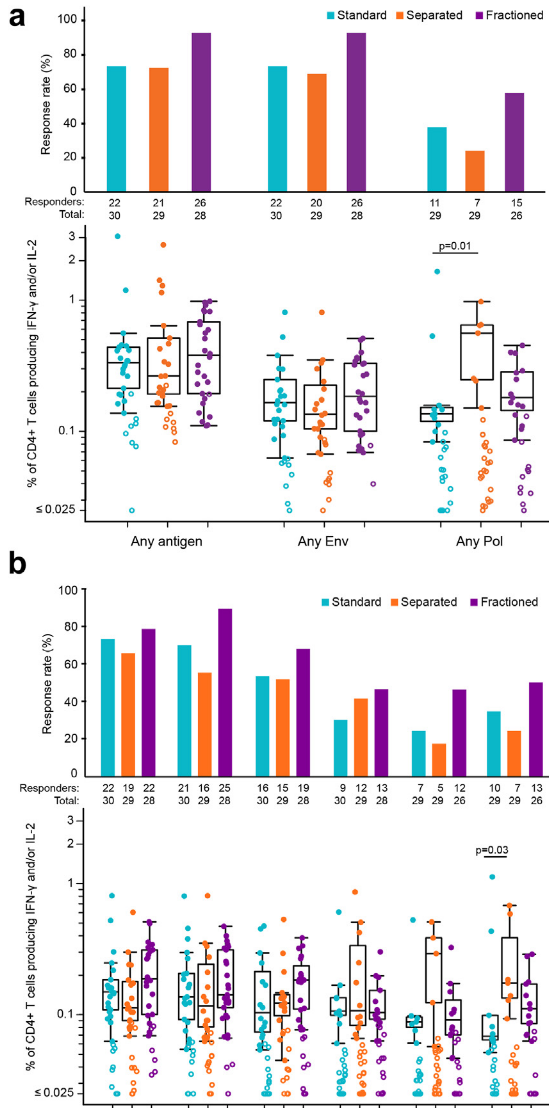

Fig. 2: **HIV antigen specific CD4+ T-cell response rates and magnitudes measured by ICS**. a). Responses to pooled antigen peptides Any antigen, Any Env and Any Pol. b). Responses to individual antigen peptides Env A, Env B, Env C, Gag, Pol 1 and Pol 2. Bar graphs represent number of participants with a positive response and box plots represent the distribution for the positive responders only, where the midline of the box denotes the median and the ends of the box denote the 25th and 75th percentiles, with whiskers extended to the extreme data points that are no more than 1.5 times the interquartile range or, if no value meets this criterion, to the data extremes. The box plots are overlaid with individual data points of both positive (filled circles) and negative responders (open circles). Per protocol-defined objectives, comparisons were done only between Standard and experimental groups. Results are from 4 weeks post vaccination. P values are Fisher's for response rates and Wilcoxon for magnitudes.

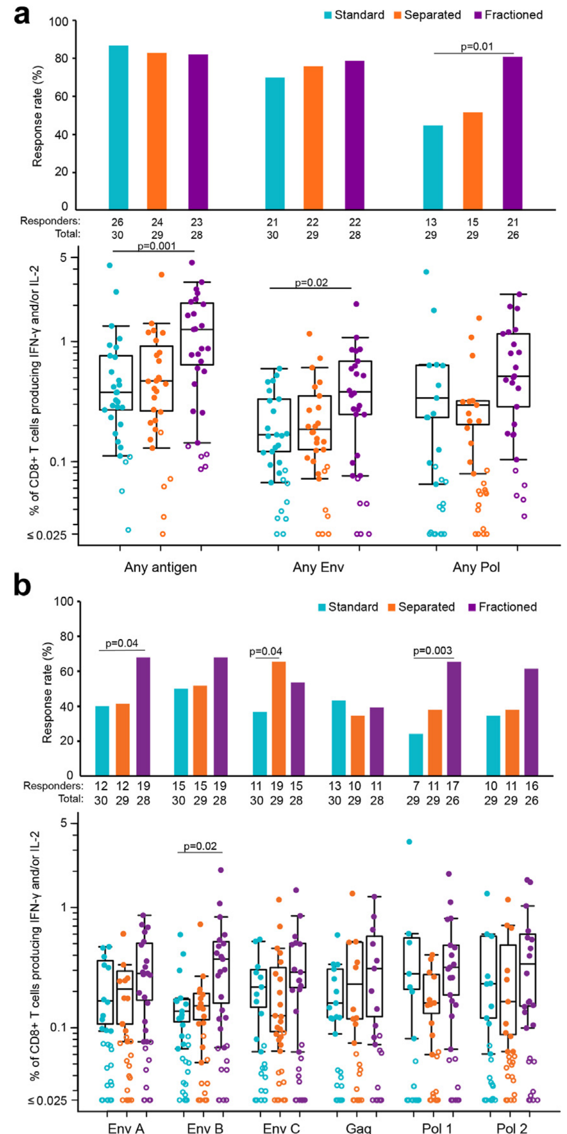

Fig. 3: **HIV antigen specific CD8+ T-cell response rates and magnitudes measured by ICS**. a). Responses to pooled antigen peptides Any antigen, Any Env and Any Pol. b). Responses to individual antigen peptides Env A, Env B, Env C, Gag, Pol 1 and Pol 2. Bar graphs represent number of participants with a positive response and box plots represent the distribution for the positive responders only, where the midline of the box denotes the median and the ends of the box denote the 25th and 75th percentiles, with whiskers extended to the extreme data points that are no more than 1.5 times the interquartile range or, if no value meets this criterion, to the data extremes. The box plots are overlaid with individual data points of both positive (filled circles) and negative responders (open circles). Per protocol-defined objectives, comparisons were done only between Standard and experimental groups. Results are from 4 weeks post vaccination. p values are Fisher's for response rates and Wilcoxon for magnitudes.

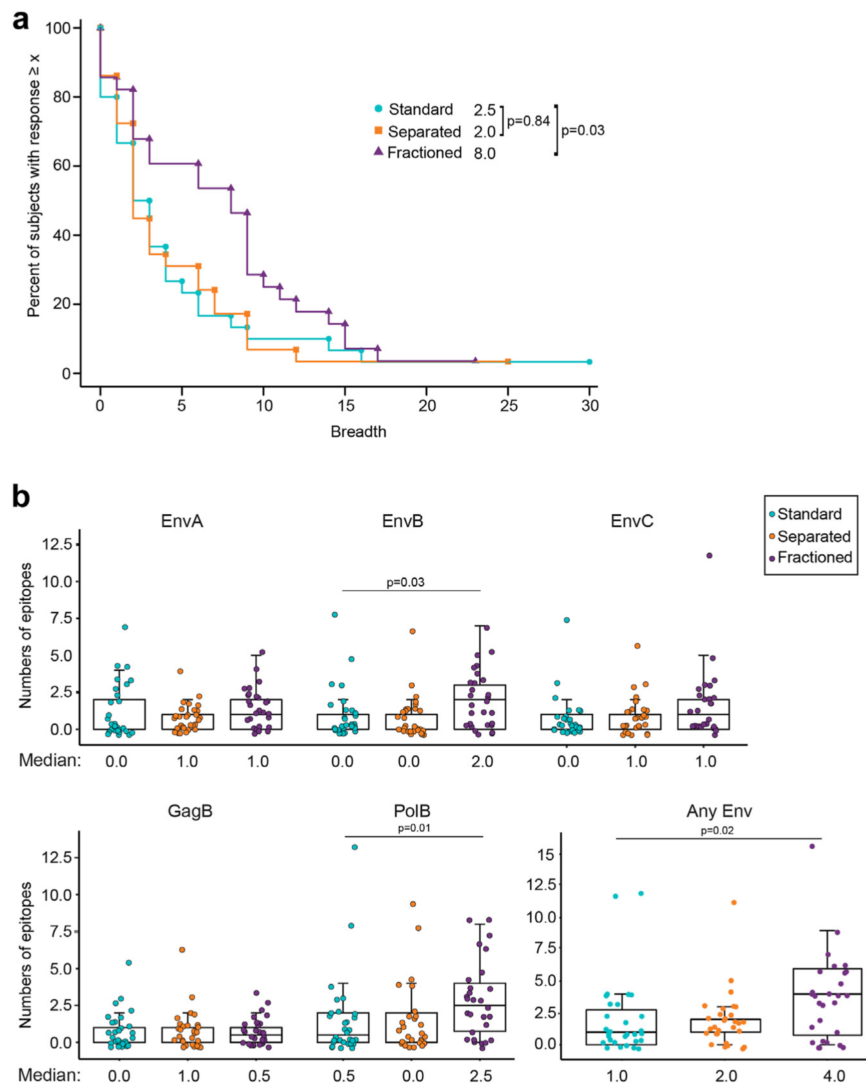

Fig. 4: **Epitope breadth of T-cell responses to HIV antigens**. Epitope mapping was performed 1 month after vaccination by IFN-γ ELISpot by deconvoluting responses to pools down to single 15-mer peptides. Responses to peptides overlapping by eight or more amino acids were counted as one epitope. a). RCDF plot showing the proportion of participants on the y axis with a response greater than or equal to the number of epitopes shown on the x axis for all antigens tested. Medians are listed beside the color labels with a p-value for Control vs Combined. b). Number of targeted epitopes to Env, Gag, Pol and Any Env induced by vaccination. The midline of the box denotes the median and the ends of the box denote the 25th and 75th percentiles, with whiskers extended to the extreme data points that are no more than 1.5 times the interquartile range or, if no value meets this criterion, to the data extremes. The box plots are overlaid with individual data points of both positive (filled circles) and negative responders (open circles). Per protocol-defined objectives, comparisons were done only between Standard and experimental groups. Results are from 4 weeks post vaccination. p values are Wilcoxon.

to specific inserts Env B (2.0 vs 0.0, Wilcoxon $p = 0.03$) and Pol B (2.5 vs 0.5, Wilcoxon $p = 0.01$). There were no significant differences in breadth between the Standard and Separated groups. The reverse cumulative distribution function (RCDF) plot showed similar participant response frequencies to 2 or fewer epitopes between the Standard and experimental groups (Fig. 4a). However, at ≥3 epitopes, the Fractioned group noticeably separated from Standard.

Epitope mapping of T-cell responses by position in each vaccine protein

To further characterize epitope breadth, we analysed the Env, Gag and Pol epitopes according to amino acid position of each vaccine protein. We then used these data for each treatment group to plot group-level frequencies of each epitope. Some common epitopes were recognized in all three groups with differing frequencies, and some were unique to specific groups

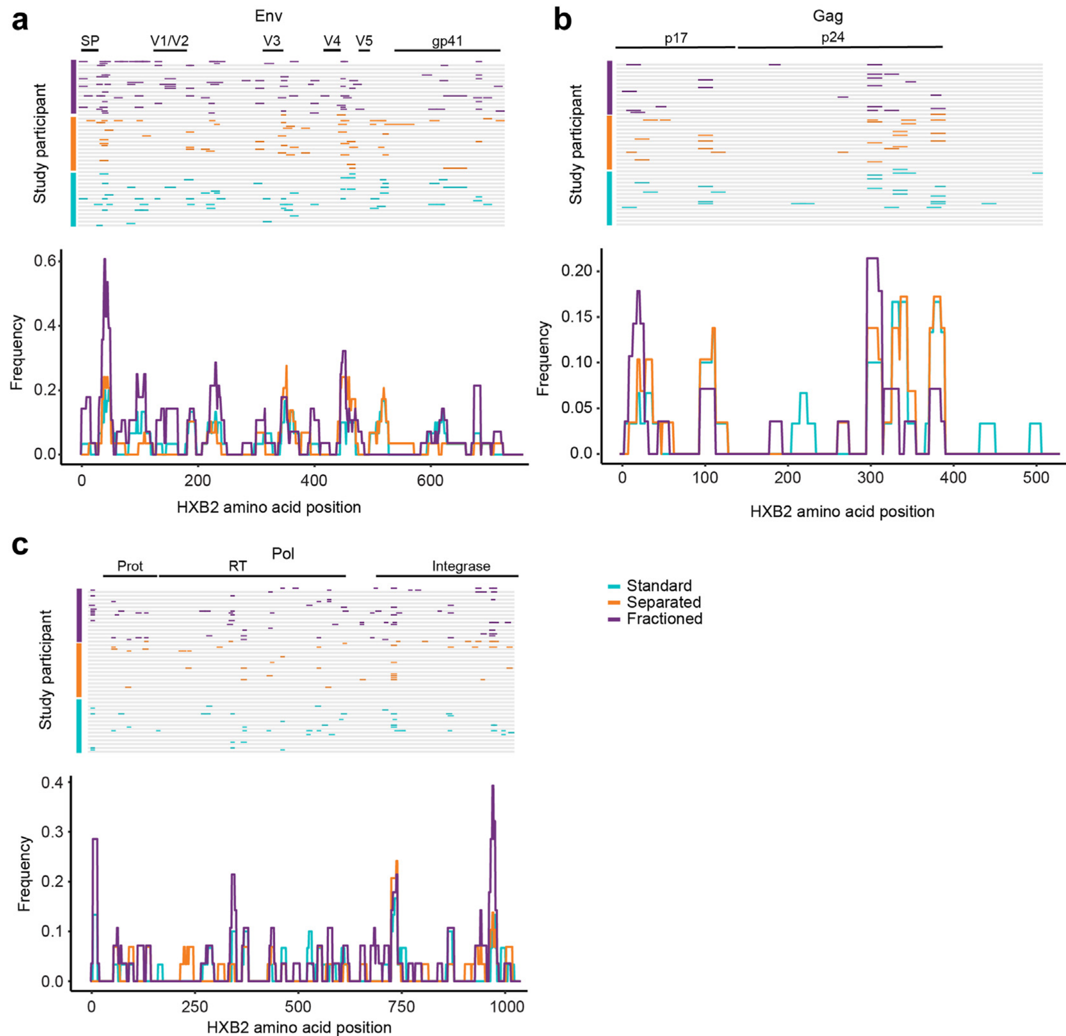

Fig. 5: **Distribution of epitopes recognized by T cells to HIV antigens**. Epitope mapping was performed 1 month after vaccination by IFN-γ ELISpot by deconvoluting responses to pools down to single 15-mer peptides. Responses to peptides overlapping by eight or more amino acids are shown as one epitope for a). Env, b). Gag, and c). Pol. Top panels: each row represents a single participant (Env, Standard n = 20, Separated n = 23, Fractioned n = 21; Gag, Standard n = 14, Separated n = 15, Fractioned n = 14; Pol, Standard n = 15, Separated n = 14, Fractioned n = 21) and empty rows represent participants for which no individual epitopes were identified. Bottom panels: number of epitopes in a group summed for total frequency in that group of an epitope being recognized.

(Fig. 5). Together with the T-cell breadth data, the diversity of T-cell responses increased with the Fractioned strategy.

There were modest positive correlations between four immunogenicity endpoints in a partial correlation analysis (Supplementary Figure S3, Supplementary Table S7), with the strongest correlation being between Env breadth and CD8+ T-cell responses (r = 0.62 [95% CI: 0.465, 0.731] $p < 0.0001$). Importantly, the strategies of separating and diluting vaccine immunogens did not affect immune response correlations.

Antibody response differences between vaccine groups

To evaluate differences in antibody responses between the three vaccine groups, IgG binding antibody frequency and magnitude were measured for Env proteins gp140, gp120 and gp41 as well as Gag protein p24 at 4 weeks post vaccination. Response rates to 4/5 (80%) gp140 antigens were significantly higher in the Standard group than Separated: 1086 trimer (69% vs 29.6%, Fisher's p = 0.007), gp140 A (67.9% vs 25.9%, Fisher's p = 0.002), gp140 B (72.4% vs 25.9%, Fisher's p = 0.001) and gp140 C (60.7% vs 18.5%, Fisher's

p = 0.001), as well as for magnitudes to ConS gp140 (Wilcoxon p = 0.0006) and gp41 (Wilcoxon p = 0.007) (Supplementary Figure S4). There were no other statistically significant differences in IgG or IgA responses between the Standard and experimental groups (Supplementary Figure S5).

Discussion

The failure of two HIV T-cell vaccines, HVTN 505 (which included the vaccine used in the current study) and Step (which included the Ad5 vector backbone with different immunogens and did not include Env), to offer protection against acquisition should be considered in the context of stimulating immune responses of narrow breadth. Whether increasing breadth of T-cell responses to vaccination can have a protective effect remains to be determined. Our results demonstrate that fractional delivery led to greater T-cell breadth than delivery to a single location, which is the standard approach for most vaccine studies. The Fractioned approach, where the vaccine containing all 5 inserts given as fractional delivery in each deltoid and each thigh, produced the greatest epitope breadth, followed by the separated approach, where each antigen was delivered in a unique location (except Gag-Pol, which were delivered together). In terms of cellular breadth, both fractional approaches were superior to standard, single site vaccination. The fractional delivery may be of some relevance to the observed discrepancy in immunological outcomes between NHP preclinical studies and subsequent trials in humans.

While previous trials have shown that single dose Ad5 vaccines result in somewhat broad cellular responses,[21,22,45] this is the only one to measure breadth based on fractional delivery of antigen. The median number of epitopes recognized by monotopic vaccination of the same rAd5-*gag-pol/env A/B/C* vaccine was approximately 2.0 in the HVTN 084 study and 3.0 in HVTN 054, compared to 2.5 seen in the current study.[21,22] However, as described above, the Fractioned group median was 8.0, which, while still lower than natural infection, was a significant increase (Wilcoxon p = 0.03). In a study of persons living with HIV from Japan, there was a positive correlation between Gag/Pol CTL epitope breadth and CD4+ T-cell counts and a negative correlation with viral load, suggesting epitope breadth is associated with control of disease progression.[46] There is evidence that increasing the breadth of cellular immune responses by only a single epitope may be clinically meaningful. In a separate study of untreated people living with HIV, an increase in the number of Gag-specific epitopes recognized (from 2 to 3) was associated with a decrease in viral load; an increase in the number of Env antigens recognized (from 0 to 3) was associated with an increase, and the number of Pol antigens recognized (0–3 compared to >3) had no effect.[47] Therefore, the specific antigen recognized, rather than number of antigens, may also be biologically relevant to the control of HIV. This hypothesis is also supported by reports of single Gag-specific escape mutations leading to progressive HIV infection or increases in HIV RNA levels.[12] Our results suggest that polytopic vaccination can induce broader cellular responses to multiple HIV vaccine antigens than single site injection, which could in theory lower the likelihood of CTL escape mutants establishing chronic infection. The HIV-Core 002 and 004 trials administered vaccine into both deltoids and showed relatively high CD8 and CD4 T-cell responses post vaccination.[36,37] While the study did not compare to single site injection, it is possible the multi-site injection contributed as least partially to the vaccination responses.

HVTN 085 was not designed to uncover the mechanism(s) by which polytopic vaccination may augment the breadth of immune responses, although several hypotheses exist. Administration of a single antigen in multiple locations may increase the chance for recognition by a variety of clones of naïve T cells with cognate T-cell receptors (TCRs) in different lymph nodes. The separation of multiple antigens through administration at multiple sites or the administration of a single antigen at multiple sites may reduce competition for major histocompatibility complex (MHC) binding on antigen presenting cells in local draining lymph nodes, particularly if epitopes share MHC class-restricted responses.[32] In humans, CD8 responses to HIV vaccines are HLA-restricted, thus individual participant genetics will influence the targeting of specific cognate epitopes.[48] As we do not have HLA typing data on these participants, we cannot address this question in the current study. Regardless, results suggest that the administration of antigens in multiple locations may increase the number and diversity of T cells exposed to cytokines that promote proliferation within local lymph nodes. Other mechanisms that regulate patterns of immune dominance may also be affected.

This study has several limitations. It was not designed to elicit high humoral responses, as it was a one dose viral vector regimen, and previous reports have shown the need for a protein boost to stimulate a robust antibody response. With the Fractioned strategy, binding antibody response rates and magnitudes were similar to the standard, single injection site approach, both being higher than antigens separated to different limbs. The Separated group consistently displayed lower response rates to Env antigens as well as lower magnitudes to gp140 and gp41. While we do not have a definite explanation of why this may have occurred, we can speculate that perhaps antibody responses to Env are elevated by the presence of Gag/Pol through increases in the diversity of antigen presentation and T-cell help in local lymph nodes; although the trial was randomized it may be subject to unmeasured random confounding. A

similar finding was seen in a nonhuman primate study comparing co-administration and contralaterally separated vaccination, with higher binding antibody responses in the co-administration group, suggesting repeat exposure to the same or similar antigens is needed for maturation of humoral responses.[35] Another limitation of this work is the vaccines were given once, as opposed to more typical heterologous prime-boost regimens that can provide significant increases in immune responses after repeat vaccinations. All assays were conducted at one timepoint post vaccination (28 days), thus they represent only a partial look at potential immune responses. And finally, the vaccine was a replication competent adenoviral vector and simultaneous dosing could affect the replication potential of the vaccine.

This report is, to our knowledge, the only HIV vaccine study in humans comparing single site and polytopic vaccination. We found distributing antigens more broadly leads to increasing the proportion of positive responders, T-cell breadth and in some cases magnitude of immune responses. This approach may be combined with prime-boosting with or without escalating doses or other means of generating an enhanced breadth and quality of T-cell response to HIV.[49,50] Going forward, HIV vaccine regimens aimed at inducing broad cellular responses may consider a polytopic strategy, especially when less invasive means of immunogen administration become more widely used.

Contributors

Conceptualization [LC, IF, NG, JK, GT, MCK], funding acquisition [LC, MJM, GT]; data analysis [AD, SCDR, AFG, PCY, NF, GT, NG, MA, MDM, BM], investigation [PS, KB, IF, RN, HVT, MCK, SK, LB], supervision [PS, KB, IF, RN, HVT, MCK, SK, LB, NG, NF, GT] writing original draft [MDM, IF], writing review & editing [MDM, AD, SCDR, AFG, PCY, NF, GT, NG, MA, PS, KB, IF, HMS, HVT, MCK, RN, SK, LB, LC, MJM, JK]. All authors confirm that they had full access to all the data in the study and accept responsibility to submit for publication. The HVTN 085 Study Team was responsible for operationalizing the trial.

Data sharing statement

All data, code, and materials used in the analysis will be provided upon publication and can be found here: https://atlas.scharp.org/cpas/project/HVTN%20Public%20Data/HVTN%20085/begin.view.

Declaration of interests

MAA is employed by NIAID, the funder of the study. HVT, KJB, SAK, AFG, LC, GDT, NF, NG, SCDR reports receiving institutional grants from the NIH. IF reports institutional grants and participation on Boards of Gilead and Viiv; LB reports institutional grants and participation on Boards of the FDA and NIAID; MJM reports institutional grants, participation on Boards of Ragon Institute, Keystone Symposia and NIH VRC, and a patent on HIV immunogens. The rest of the authors as well as HVTN 085 Study Team declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2024.104987.

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