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HIV-1 vaccine-induced immunity in the test-of-concept Step Study: a case-cohort analysis

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Abstract

Background—In the Step Study, the MRKAd5 HIV-1 gag/pol/nef vaccine did not lower post-infection plasma viremia, and HIV-1 incidence was higher in vaccine-treated than placebo-treated males with pre-existing adenovirus serotype 5 (Ad5) immunity. We evaluated vaccine-induced immunity and its potential contributions to infection risk.

Methods—To assess immunogenicity, HIV-specific T-cells were characterized *ex vivo* using validated IFN- γ ELISpot and intracellular cytokine staining (ICS) assays, employing a case-cohort design. To determine effects of vaccine and pre-existing Ad5 immunity on infection risk, flow cytometric studies measured Ad5-specific T-cells and circulating activated (Ki67+/Bcl-2^{lo}) CD4+ T-cells expressing CCR5.

Findings—IFN- γ -secreting HIV-specific T-cells (range, 163–686/10⁶ PBMC) were detected *ex vivo* by ELISpot in 77% (258/354) of vaccinees; the majority recognized 2–3 HIV proteins. HIV-specific CD4+ T-cells were identified by ICS in 41%; ~85% expressed IL-2, and two-thirds of these

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Conflict of Interest

SD, LK, MNR, DVM, JWS, and DRC all are employees and shareholders of Merck & Co, Inc. MJM and SPB have served as investigators on Merck-funded research, SCD, ZM, HJ, ODD, DKC, JH, RA, SGS, and LC have no conflicts of interest.

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co-expressed IFN- γ and/or TNF- α . HIV-specific CD8+ T-cells (range, 0.4–1.0%) were observed in 73%, expressing predominantly either IFN- γ alone or with TNF- α . No major differences were found in vaccine-induced HIV-specific immunity, including response rate, magnitude, and cytokine profile comparing vaccinated male cases (pre-infection) with non-cases. Interestingly, Ad5-specific T-cells were lower in cases than non-cases in several subgroup analyses. The percent circulating Ki67+Bcl-2^{lo}/CCR5+ CD4+ T-cells did not differ between cases and non-cases.

Interpretation—Consistent with previous trials, the MrkAd5/HIV-1 gag/pol/nef vaccine was highly immunogenic for inducing HIV-specific CD8+ T-cells. Comparative analyses did not reveal differences in HIV-specific immunologic responses between cases and non-cases that explain the lack of vaccine efficacy and potential infection enhancement. If T-cell immunity is critical in vaccine-induced HIV protection, our findings suggest that future candidate vaccines must elicit responses that either exceed in magnitude or differ in breadth and/or function from those observed in this trial.

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Introduction

The development of a safe, effective HIV vaccine is one of the world's highest public health priorities. Over the past decades, more than 50 candidate vaccines, designed to elicit either HIV-specific antibodies and/or T cells, have progressed to clinical trials based upon promising preclinical evaluation (1,2). Only two regimens, recombinant gp120 subunit alone and as a boost following canarypox/HIV priming, have proceeded to large scale testing (3–5). Two international trials with the envelope subunit alone have failed to show protective efficacy, and the prime-boost study is still underway. More recent attention has focused on immunogens that can generate long-term memory CD8+ T cells that recognize conserved viral epitopes. This strategy potentially arms the host with immunity that controls plasma viremia and disease, and indeed, in non-human primate models can significantly reduce viremia and improve survival after homologous viral challenge (6–8). The most potent candidates for eliciting such responses have been the recombinant, replication-incompetent adenovirus serotype 5 HIV vaccines (9, 10), which led to their assessment in advanced clinical trials.

The Step Study, initiated in late 2004, was the first phase IIB test-of-concept trial to determine if the MRKAd5 HIV-1 gag/pol/nef vaccine expressing subtype B HIV-1 Gag, Pol and Nef in a three-dose regimen could lower either HIV-1 infection rates or plasma viremia post-infection in 3000 subjects potentially exposed to circulating subtype B viral strains. A planned interim analysis was conducted when the number of per-protocol infections reached 30 in subjects whose baseline Ad5 neutralizing titers were ≤ 200 . This review (11), demonstrated no current evidence or future likelihood for vaccine efficacy. Surprisingly, risk for infection was highest in the subgroups of vaccine-treated males who were both uncircumcised and had pre-existing Ad5 neutralizing antibodies when compared to the placebo control cohort; risk was intermediate in those with either one of these two factors (11). As a consequence, study participants were unblinded to their treatment assignment, were counseled regarding the study findings and HIV risk reduction, and received no further immunizations. Most volunteers are continuing into longitudinal safety and immunogenicity monitoring.

Although the study outcomes may never be fully explained, the Step study raises fundamental scientific questions that are crucial to address in order to move the HIV vaccine field forward, particularly in discerning if T cell-based vaccines hold promise as protective HIV immunogens. Two major questions are why the study failed to show efficacy and why infection rates apparently increased in uncircumcised vaccinated men with pre-existing Ad5 immunity. We launched a series of hypothesis-driven studies using sophisticated immune-based technologies in a case-cohort design in vaccinated males to gain insight into underlying mechanisms for

why the vaccine did not lower infection rates and may have enhanced infection in certain male subgroups. More comprehensive analyses, not presented here, are underway as additional cases accrue to discern why vaccine did not lower viral load set point.

Our initial studies used a validated IFN- γ ELISpot assay (12) to determine if the vaccine elicited the expected strong immunogenicity as observed in previous phase I clinical trials. Using a validated multicolor intracellular cytokine staining assay (13), we next addressed the hypothesis that the characteristics of vaccine-induced T cell immunity to the HIV insert were different in response frequency, magnitude or function between male vaccine recipients who subsequently acquired HIV infection (cases) versus matched controls who remained uninfected (non-cases). To understand the apparent increased infection rates, we hypothesized that vaccine may have heightened susceptibility to HIV-1 infection in males with preexisting Ad5 immunity by activating vector-specific CD4+ T cells. In addition to determining the response frequencies of Ad5-specific CD4+ T cells, we examined CD4+ T cells for immune activation and increased expression of the HIV-1 coreceptor, CCR5, by multiparameter flow cytometry in cases and non-cases.

Methods

Step study design and vaccine

The Step Study, so named to signify a step forward to developing an HIV vaccine, was a “test of concept” phase IIB multicenter double-blind, randomized, placebo-controlled study of the MRKAd5 HIV-1 gag/pol/nef vaccine in HIV-1 negative individuals at high risk of HIV-1 acquisition (11). The criteria for inclusion into the protocol and study design details have been described (11). The trial enrolled 3000 subjects in nine countries where clade B is the predominant subtype, with half having pre-enrollment Ad5 neutralizing antibody titers ≤ 200 and the other half with titers > 200 . Ad5 neutralizing antibody titers were measured by a previously described method (14), with ≤ 18 indicating undetectable, 19–200 indicating low, 201–1000 indicating medium and > 1000 indicating high titer responses. The institutional human subjects review committee at each clinical site approved the protocol prior to study initiation, and participants completed a thorough written informed consent process before study enrollment.

The vaccine was a mixture of three E1-deleted recombinant Ad5 viruses, each containing one of three HIV-1 inserts (HIV-1_{CAM-1} *gag*, HIV-1_{IIIB} *pol*, and HIV-1_{JR-FL} *nef*) under the control of the hCMV IE promoter and the bovine growth hormone polyadenylation sequence. Vaccine was administered intramuscularly as a 1.0 ml injection of 1.5×10^{10} adenovirus genomes, equivalent to the 3×10^{10} viral particle dose used in previous vaccine trials (9,10), at day 0, week 4 and week 26. The *pol* gene consisted of coding sequences for reverse transcriptase and integrase, whose gene products were inactivated by replacing the active acidic residues with alanines; Nef was inactivated by altering the glycine myristoylation site. The placebo contained the vaccine diluent only.

Peripheral blood mononuclear cells (PBMC) were isolated from EDTA-anticoagulated blood obtained at weeks 8 (4 weeks after the second dose), 30 (4 weeks after the third dose), 52 and 104, and cryopreserved within 12 hours of venipuncture, using previously described procedures (15). Comprehensive quality procedures were utilized to ensure the highest function and optimal recovery of PBMC. The assessment and diagnosis of new HIV-1 infection and measurement of plasma HIV-1 RNA were implemented as previously described (11).

Study population for laboratory analyses

Laboratory analyses were conducted in defined subgroups, depending upon the nature of the study and the availability of PBMC. The original protocol specified performance of immunogenicity analyses on 25% of study participants, stratified on treatment status and study site, to compare findings with previous phase I studies. This subgroup was designated the stratified random sample (SRS). Investigations examining vaccine-induced responses in persons who acquired infection (cases) were restricted to the per-protocol (PP) population (11), which included all subjects who received at least the first two doses of either vaccine or placebo before being diagnosed with HIV-1 infection (i.e., remained uninfected through week 12) and/or were identified as protocol non-violators based on pre-defined criteria. The PP population was selected to ensure that all subjects analyzed were HIV-uninfected at week 8 and that immunogenicity endpoints reflect at least two injections. As of Oct 17, 2007, 59 per-protocol (PP) participants acquired HIV-1 infection; all but one was male, 38 received vaccine and 20 received placebo (11).

The IFN- γ ELISpot assay (Table 1) was performed on week 8 PBMC from subjects in the SRS non-cases (including males and females) and PP cases. Findings were compared with study participants receiving the same vaccine dose in a previous phase I trial whose design and subject enrollment criteria have been previously described (10). The PP vaccinated males who became HIV-1 infected served as the cases for further analysis of antigen-specific T cell responses by intracellular cytokine staining of HIV-specific (Figures 1 and 2, Table 2) and Ad5-specific T cell responses (Figure 3). Two to four male non-cases were frequency matched to the cases based on treatment, baseline Ad5 antibody titer (≤ 18 , 19–200, 201–1000, >1000), region (North America/other), circumcision status, and time of specimen collection (week 8 or week 30). Non-cases were preferentially sampled from the SRS described above. Flow cytometric studies of T cell activation (Figure 4) were also performed on PP cases and primarily SRS non-cases, selected on the basis of PBMC availability.

Immunological assays

Validated IFN- γ ELISpot assays (14) were conducted using previously cryopreserved PBMC obtained at week 8, stimulated *ex vivo* with pools of peptides 15 amino acids in length and overlapping in sequence by 11 amino acids. The peptide sequences were based upon matched HIV-1 proteins encoded by the vaccine, with four total pools of peptides: one Gag pool, two Pol pools, and one Nef pool. Responses were measured as the number of spot-forming cells per million PBMC and expressed as geometric means; the criteria for positive and negative responses were defined in a previous publication (12).

Intracellular cytokine staining (ICS) assays were performed by flow cytometry using previously cryopreserved PBMC to determine both HIV-specific and Ad5-specific CD4+ and CD8+ T cell responses. PBMC were obtained at week 30 from cases infected after week 30, and at week 8 from cases infected between weeks 12 and 30; PBMC from non-cases were also obtained at the same time points to match cases for comparative analyses. For the detection of HIV-specific T cells, thawed PBMC were cultured overnight and then stimulated for six hours with the same HIV-1 peptide pools as for ELISpot. For the detection of Ad5-specific T cells, an “empty” vector lacking HIV-1 gene inserts (kindly provided by Dr. Gary Nabel, NIH Vaccine Research Center, Bethesda, MD) was used to stimulate PBMC. The Ad5 vector was added to PBMC at a ratio of 10,000 Ad5 particle units per cell, six hours later the cells were treated with Brefeldin A, and the ICS assay was performed after an overnight incubation. The 8-color ICS protocol was previously validated (13) for detection of *ex vivo* IFN- γ - and IL-2-secreting CD3+/CD8+ and CD3+/CD4+ HIV-specific T cells. In addition, both TNF- α and perforin expression were examined, with the latter replacing the APC IL-4 that was used in the original protocol. The perforin antibody (Tepnel/Diaclone, Stamford, CT) was conjugated to

Alexa 647 (Invitrogen, Eugene, OR) in the laboratory. An alternate 10-color ICS assay also was used for analysis of cytokine expression, which included an evaluation of granzyme B and CD57 expression. IFN- γ and IL-2 expression determined by the 10-color assay was validated in bridging studies with the 8-color assay, and results for samples analyzed with the two assays are shown in Figures 1 and 3. Of note, TNF- α intracellular expression was not cross-validated between the two assays. Therefore, only data from the 8-color assay are shown in Figure 2.

To assess T cell activation, immediately thawed PBMC were first stained with Aqua Live/Dead Fixable Dead Cell Stain (Invitrogen, Eugene, OR) (16), and then surface stained with anti-CCR7 PE-Cy7, anti-CCR5 PE-Cy5, anti-CD27 APC-Alexa750, anti-CD38 APC and anti-HLA-DR Pacific Blue. Cells were fixed and permeabilized as for the 8-color ICS assay (13), followed by an intracellular stain with anti-CD3 PE-TR, anti-CD4 Alexa700, anti-CD8 PerCP-Cy5.5, anti-Ki-67 FITC and anti-Bcl-2 PE. All reagents were obtained from BD Biosciences (San Jose, CA), except for anti-CD3 (Beckman Coulter, Miami, FL), anti-CD27 (eBioscience, San Diego, CA) and anti-HLA-DR (Biolegend, San Diego, CA).

For all flow cytometric analyses, the specimens were collected from 96-well plates using the High Throughput Sample (HTS, BD) device and analyzed with a BD LSRII using FlowJo software (Treestar, Inc; OR) or LabKey Flow (17). Positive responses and criteria for evaluable responses were determined as previously described (13), and were based on background measurements and number of T cells examined. Since separate criteria are applied for CD4+ and CD8+ cells, the total numbers included in each ICS analysis can differ between the CD4+ and CD8+ T cell evaluations.

Statistical analyses

The magnitudes of immune responses are displayed with boxplots, using colors to distinguish between positive and negative responders and shapes to designate week 8 and week 30 samples. Each response is identified using a point on the plot; the boxplots show the distribution of responses among positive responders only. The box indicates the median and interquartile range; whiskers extend to the furthest point within 1.5 times the interquartile range from the upper or lower quartile. Response rates were compared using logistic regression. Confidence intervals for response rates were calculated using the method of Agresti and Coull (18), and ninety-five percent confidence intervals are reported in the text. Log-transformed magnitudes of response among positive responders were compared using linear regression. Tests were adjusted for treatment and Ad5 titer (≤ 18 , >18) when these factors were not the predictors of interest, as well as for region (North America/other), circumcision status, and time of specimen collection (week 8 or week 30). No adjustment was made for multiple testing.

Role of the funding source

This study was funded by Merck Research Laboratories; the Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID), in the U.S. National Institutes of Health (NIH). Each partner contributed to the study design, the data collection and analysis, and the manuscript preparation and submission. The immunologic studies were performed at the Merck Research Laboratory and the HIV Vaccine Trials Network Laboratory Program.

Results

Screening immunogenicity studies and comparison of results to previous phase I trials

To define the immunogenicity of the MRKA₅ HIV-1 trivalent vaccine, an ELISpot assay was performed to detect *ex vivo* IFN- γ -secreting T cells from previously cryopreserved PBMC obtained at week 8 from 354 vaccine recipients, including 316 non- cases in the stratified random sample and 38 vaccine-treated PP cases. As shown in Table 1, high response

frequencies were observed after two immunizations in the vaccine recipients, particularly in the undetectable (≤ 18) or low (≤ 200) Ad5 groups. Overall, the vaccine elicited HIV-specific T cells recognizing one or more gene product in 86% (CI: 79–91%) of low Ad5 (≤ 200) and 68% (CI: 61–75%) of high Ad5 (>200) non-case subjects. The magnitude of responses, as indicated by geometric means, was greater for the Pol than for the Gag and Nef peptide pools, regardless of pre-existing Ad5 titer. Prior Ad5 immunity did reduce the immunogenicity of the vaccine; 58% (CI: 50–66%) of Ad5 ≤ 200 non-case vaccine recipients, but only 34% (CI: 27–41%) of Ad5 >200 non-case vaccine recipients, manifested responses to all three HIV-1 gene products (Table 1).

Since the Step Study failed to demonstrate vaccine efficacy, determining if the level of immunogenicity induced in the phase IIB trial was similar to previous phase I trials was of considerable concern. Therefore, responses of 35 subjects receiving the same dose and regimen of the same vaccine in an earlier multicenter phase I trial (10) were compared with 354 uninfected subjects in the Step trial at the week 8 time point. For this comparison, non-case groups were stratified by Ad5 titer (≤ 200 or >200 as in the original trial designs). We found that the two studies did not differ in response frequencies (range, 33–100% in phase I vs. 47–76% in Step) and geometric means recognizing any of the three gene products (range, 90–202 SFC/ 10^6 PBMC in phase I vs. 163–686 SFC/ 10^6 PBMC in Step). Confidence intervals for response rates to the three gene products easily overlap (data not shown). Thus, the immunization regimen in the Step trial provided the expected level of immunogenicity among persons with low and high Ad5 titers, indicating that vaccine potency was retained and discounting the possibility that a weakened vaccine product could explain the Step efficacy results.

Vaccine induction of HIV-specific T cells in cases and non-cases

To address the hypothesis that lack of vaccine efficacy was associated with suboptimal HIV-specific T cell responses in cases in comparison to non-cases, we first examined response rates and magnitude using the screening IFN- γ ELISpot assay. Of note, 71% (CI: 55–83%) of cases mounted IFN- γ -secreting HIV-specific T cells, similar to 76% (CI: 71–81%) of those who did not become infected as detected by IFN- γ ELISpot. Moreover, the geometric mean responses to each gene product were greater in cases than in the non-cases (Table 1). As noted in the non-cases above, the cases in the Ad5 ≤ 200 (and Ad5 ≤ 18) subgroup had stronger response frequencies and magnitudes to each gene product than cases with Ad5 >200 (and Ad5 >18). These data suggest that induction of IFN- γ -secreting T cell responses by vaccine was not impaired among those who acquired HIV-1 infection during the study.

To ascertain if the candidate HIV vaccine induced HIV-1 specific immunological memory in both CD4+ and CD8+ T cells, and if there were distinguishing functional features in cases vs. non-cases, we performed multiparameter flow cytometric analyses to identify cytokine-expressing T cells that recognized epitopes expressed by the HIV-1 gene inserts. The measurements were conducted on previously cryopreserved PBMC obtained at weeks 8 or 30 in 30 male vaccinated cases and 130 matched non-cases, depending upon the time of HIV-1 infection in the cases (see methods), and included intracellular expression of IFN- γ , IL-2 and TNF- α in ex vivo CD3+CD4+ and CD3+CD8+ T cell populations. As with the ELISpot assay, overall, 77% (24/31; CI: 60–89%) of cases and 76% (101/133; CI: 68–82%) of non-cases mounted an HIV-specific T cell response after two or three immunizations using the validated ICS assay. The results from a representative vaccine recipient are depicted in Figure 1A. After three immunizations, this subject mounted an easily distinguishable Gag-specific CD4+ T cell response, with populations secreting predominantly IL-2 alone or in combination with IFN- γ and TNF- α . By contrast, the CD8+ T cell response in this subject was Pol-specific, with

populations expressing predominantly IFN- γ alone or in combination with IL-2 and TNF- α (Figure 1A).

Among the male vaccine recipients (regardless of Ad5 titer and HIV-1 infection) tested, 46% (CI: 29–65%) of cases and 40% (CI: 31–49%) of non-cases generated an HIV-specific CD4+ T cell response after two or three immunizations (Table 2), and 55 of the 58 responders recognized epitopic peptides within the Gag pools. By contrast, CD4+ T cells directed to Pol and Nef were less common in both cases and non-cases (Table 2). Of interest, the percentage of positive responders did not differ significantly between cases and non-cases within the Ad5 ≤ 18 group ($p=0.84$) and the Ad5 >18 group ($p=0.65$) (Table 2). The median magnitude of responding CD4+ T cells ranged from 0.22–0.26% (Figure 1B) and did not differ among positive responders between the Ad5 ≤ 18 or >18 groups, stratified by case vs. non-cases ($p=0.70$ and 0.82 , respectively). Similar patterns were noted in the Gag-specific T cell responses in cases vs. non-cases (Figure 1B).

In contrast to the CD4+ T cell responses, the vaccine elicited a substantially higher CD8+ T cell response rate and magnitude. Among the male vaccine recipients, 73% generated HIV-specific CD8+ T cells secreting IFN- γ and/or IL-2, and the predominant response was directed to HIV-1 Pol (Table 2). Gag-specific CD8+ T cells were less commonly elicited overall (40% in cases, 42% in non-cases), particularly in the Ad5 >18 group (29% in cases, 25% in non-cases) (Table 2). The effect of previous Ad5 immunity was more apparent in the percentage of CD8+ responders than the CD4+ responders (see above); those with Ad5 ≤ 18 titers exhibited a higher probability of response than those with the higher baseline Ad5 titer >18 (Odds ratio = 5.76, $p=0.0006$). The magnitude of HIV-specific CD8+ T cell responses (median 0.4–1.0%) was typically greater than the CD4+ T cell responses, and in some cases was up to 12% of circulating CD3+CD8+ T cells (Figure 1C).

Thus, the trivalent HIV-1 vaccine elicited easily detectable peak T cell responses after two or three doses, including T helper responses in more than a third of male vaccine-recipients. While the overall frequency of T cell responses was high, only 28% of vaccine recipients (CI: 18–42%) mounted both CD4+ and CD8+ HIV-specific T cell responses after two doses and 31% (CI: 24–41%) mounted both after three doses. Those without previous Ad5 vector immunity had significantly higher CD8+ T cell response rates. In addition, we observed no significant ($p \leq 0.05$) differences between the cases and matched non-cases in their ability to mount either a positive CD4+ or CD8+ HIV-specific T cell response or in the magnitude of these responses.

Analysis of polyfunctional antiviral T cell responses in cases and non-cases

To determine if the antiviral properties of the HIV-specific T cells were impaired in cases compared with non-cases, we next examined by 8-color flow cytometry the capacity of CD4+ and CD8+ T cells to produce intracellularly the Th1-type antiviral cytokines IL-2, TNF- α and/or IFN- γ , in response to the HIV-1 antigens expressed in the vaccine insert (Figure 2). As no differences were observed in the cytokine profiles by Ad5 titer groups or by gene product, summary data are provided here for the overall subjects and the HIV-specific responses. Among those mounting an HIV-specific CD4+ T cells to Gag, Pol or Nef after three immunizations, approximately one-third of these cell populations produced either one, two or three cytokines (Figure 2A). IL-2 was the major cytokine expressed, comprising approximately 88% of the HIV-specific CD4+ T cells detected, and of these, approximately two-thirds produced either TNF- α and/or IFN- γ (Figure 2A). Similar profiles were noted for populations specific for Gag, Pol and Nef (not shown). The patterns of cytokine production by CD4+ T cells were remarkably comparable between the cases vs. non-cases (Figure 2A).

The quality of the HIV-specific CD8+ T cell vaccine-induced responses was distinct from the CD4+ T cell responses. The majority of CD8+ T cells produced two cytokines, and 10–20%

produced three antiviral cytokines (Figure 2B). Clearly, IFN- γ was the predominant cytokine in the single- and dual-producing populations (in combination with TNF- α), whereas IL-2-producing cells were infrequently detected (Figure 2B). Again, no cytokine profile distinguished the responding populations of the male cases vs. non-cases who received vaccine (Figure 2B) that could explain the risk of infection in the vaccinated cases.

Induction of Ad5-specific T cells

With the observation that baseline Ad5 immunity was a potential risk factor for increased infection in male vaccine recipients relative to placebo recipients, we hypothesized that T cell immune responses to the Ad5 vector itself may have influenced the immune response to the HIV-1 insert and/or increased the influx of target cell populations with increased susceptibility to HIV-1 infection. While this complex issue cannot be fully addressed with available reagents and PBMC alone from the Step Study, we characterized Ad5-specific circulating CD4+ and CD8+ T cells detected by *ex vivo* stimulation with an empty Ad5 vector (lacking the HIV-1 insert) and multicolor ICS assay for cases and matched non-cases who were vaccine recipients (Figure 3). A representative response in a vaccine recipient at week 30, whose baseline Ad5 titer was 893, is depicted in Figure 3A.

After two or three immunizations, circulating adenovirus-specific CD4+ T cells were detected in most vaccine recipients (54–96%), regardless of their entry Ad5 Ab neutralizing titer (Figure 3B). The median magnitude of the adenovirus-specific CD4+ T cells ranged from 0.2–0.3%, which was similar to that of the HIV-specific response in the vaccinated group (Figure 1B, median response, 0.2 to 0.3%). Of interest, adenovirus-specific CD4+ T cell responses were less frequent among cases than non-cases after vaccination. Examining this effect by Ad5 titer, a lower CD4+ T cell response rate was observed in cases with baseline Ad5 titer ≤ 18 in comparison to matched non-cases (71% vs. 96%, respectively). This difference was statistically significant after adjusting for covariates using logistic regression (odds ratio = 0.038; $p = 0.03$). Likewise, response rates were lower in the cases with Ad5 > 18 (where excess risk was observed) than non-cases (54% vs. 74%, respectively), but this association was not statistically significant after adjusting for covariates (odds ratio = 0.402, $p = 0.16$).

Adenovirus-specific CD8+ T cells were also identified in the majority of vaccinated recipients (Figure 3C). The median magnitude of responses was lower (0.2 to 1.0%) than those observed for the HIV insert (Figure 1C, median magnitude, 0.4 to 1.0%), but were similar in magnitude to the CD4+ Ad5-specific responses. Significantly lower CD8+ T cell response rates were also observed for Ad5 > 18 cases as compared to matched non-cases (Odds ratio = 0.198, $p = 0.02$). The association was in the same direction but not statistically significant among cases and non-cases with Ad5 ≤ 18 (Odds ratio 0.231, $p = 0.14$). Taken together, these findings suggest that cases were less likely than non-cases to direct T cell responses to whole Ad5 antigens that can be detected in peripheral blood. Analysis with Ad5 peptide pools will be important to confirm and further explore these results.

CD4+ T cell activation and CCR5 expression

To address the possibility that vaccination rendered target cells, particularly CD4+ T cells, more susceptible to HIV-1 infection, we evaluated freshly thawed PBMC for the surface expression of phenotypic markers that commonly signify T cell activation (Ki67^{hi}BcL-2^{lo}) as well as CCR5, the receptor that serves as the major path of entry of most sexually-acquired HIV-1 infections (see Figure 4A for representative staining). The first investigations sought to identify potential differences in the percentage of activated CD4+ T cells expressing CCR5 in vaccine vs. placebo recipients. Approximately 30–45% (median) of activated (Ki67^{hi}BcL-2^{lo}) CD4+ T cells from study participants expressed CCR5+, and this proportion was unrelated to treatment (vaccine or placebo) when assessed at week 30 (Figure 4B) and

week 52 (Figure 4C). Similarly, and more importantly, no evidence of increased CCR5+ activated T cells was found when comparing cases with non-cases at week 30, after the full course of immunization (Figure 4B).

Because risk of infection was more apparent in the vaccine subgroup with Ad5 immunity, further assessments of T cell activation were explored among Ad5 subgroups (≤ 200 vs. >200 or ≤ 18 vs. >18). At week 30 in both vaccine and placebo recipients, non-case study participants with baseline Ad5 titers >200 demonstrated a significantly greater median percentage of activated CCR5+CD4+ T cells at the peak of immunization (week 30) than the group with Ad5 titers ≤ 200 (Figure 4B). After adjustment for region and circumcision, this difference was statistically significant among placebo recipients ($p = 0.004$) but not among vaccine recipients ($p = 0.30$). The trend toward higher levels of activated CCR5+CD4+ T cells among non-cases with Ad5 >200 persisted at week 52 among those who remained HIV-1 uninfected (Figure 4C) ($p = 0.07$ for placebo recipients, $p = 0.13$ for vaccine recipients). A similar but less striking pattern was found when comparing by Ad5 titers ≤ 18 vs. >18 at weeks 30 and 52 (not shown).

Discussion

From our comprehensive immunologic analysis of the Step trial, several key findings emerge that bear relevance to the trial outcome and future HIV vaccine design. We demonstrate that the MRKAd5 HIV-1 gag/pol/nef vaccine elicited a higher CD8+ T cell response rate and magnitude than that reported for any of the candidate immunization regimens tested over the past 15 years (1), although immunologic assays have changed considerably over this period of time. Furthermore, pre-existing Ad5 immunity substantially influenced responses to the vaccine antigens. The potency of HIV-specific CD8+ T cells and the antiviral cytokines they elaborated in vaccinated cases were similar to their matched non-cases. These findings suggest two possible explanations for the disappointing trial results: 1) the characteristics of T cell immunity that might afford HIV-1 protection must be more broadly reactive or qualitatively different than those elicited by this vaccine, or 2) immune responses mounted by T cell-based vaccines alone will not be sufficient to protect against HIV infection or disease. We view that, before concluding the latter, we must exclude the possibility of the former as feasible both in the Step study and future related preclinical and clinical trials.

Since 77% of vaccinated cases generated HIV-specific T cells prior to infection, obviously their mere presence was not sufficient for protection. The first consideration is that the magnitude of response was too low, particularly if a substantial proportion of effectors must migrate to mucosal sites. In Figure 1, approximately 0.5–1% (in some up to 12%) of circulating CD8+ T cells were HIV-specific at a peak time point prior to infection in cases. Conceivably, a threshold level may be necessary following immunization to provide a recall response that can efficiently control early bursts of replication following HIV exposure. The median percentage of CD8+ T cells induced by the MrkAd5/HIV-1 vaccine is 43% lower than that observed in our investigations of CD8+ T cells in Seattle long-term non-progressors evaluated with the same assay using peptide reagents covering the three gene products (manuscript in preparation). Whether this quantitative difference is a major contributor to the vaccine's lack of efficacy is uncertain, and may be particularly relevant in persons with pre-existing Ad5 immunity whose magnitudes and response frequencies were lower than those without previous immunity. However, the extrapolation of immune factors associated with control of chronic HIV-1 infection may be misleading as a model for predicting risk of infection, and the magnitude of IFN- γ -secreting T cells has not been shown to correlate with contemporaneous viral load in acute infection (19,20). Hence, to inform future T cell-based HIV vaccine design, non-human primate vaccine studies could provide additional insight into whether a threshold quantity of CD8+ T cells exists, regardless of specificities, in order to substantially lower viral load during acute infection.

One leading hypothesis for the lack of efficacy of the MrkAd5/HIV-1 vaccine is that HIV-specific CD8+ T cells generated in cases lacked sufficient breadth to recognize epitopes within the transmitting viral strains. Thus, even if the quantity of HIV-specific CD8+ T cells was adequate, their specificities may have been too narrowly focused on a few epitopes that are distinct from the corresponding sequences within the transmitting strains. (21,22). Thus, vaccines that induce CD8+ T cells that recognize multiple diverse epitopes, as demonstrated in some SIV vaccine models (23,24), may hold more promise in containing the spread of the heterologous transmitting HIV-1 strain. Our comprehensive evaluation of vaccine-induced T cell determinants and transmitting viral sequences in the cases will shed light on this possibility.

An alternative explanation for the lack of vaccine efficacy is that the antiviral function of the T cell effectors was incapable of controlling viremia, particularly at mucosal sites. We demonstrated that most vaccine-induced CD8+ T cells produced IFN- γ alone or in combination with TNF- α ; both cytokines have antiviral activities that can mediate clearance of some infections (25,26). Only a small percentage of HIV-specific CD8+ T cells expressed IL-2. Of note, in natural HIV-1 infection, “protective” CD8+ T cells have been associated with antigen-specific proliferation (27), commonly secrete IL-2 (28,29) and have been linked to perforin expression (30). The proliferative capacity and expression of cytolytic granules will be evaluated in the vaccine-induced CD8+ T cells to determine the possibility of functional impairment. Further, the possibility that a skewed functional profile may have resulted from repeated immunizations is also a concern, one that cannot be addressed in the Step study but can in future clinical trials by varying vaccine dose.

One longstanding concern has been that immunization with MrkAd5 HIV-1 gag/pol/nef vaccine alone may not optimally prime CD4+ helper cells, which are important in maintaining long-term antiviral CD8+ T cell memory. Here we found that the vaccine elicited HIV-specific CD4+ T cells with a Th1-type cytokine profile, similar to those memory CD4+ T cells associated with vaccine-mediated protection in animal models and in successful control of HIV-1 and other chronic infections ((31) and reviewed in (32)). In the Step study, approximately 85% of CD4+ T cells secreted IL-2; of these, approximately two-thirds also produced TNF- α and/or IFN- γ . However, the vaccine induced HIV-specific CD4+ T cell responses in just 41%, and only 31% mounted both CD4+ and CD8+ HIV-specific T cells after the full immunization series. Importantly, both cases and non-cases mounted similar response rates and magnitudes. These findings suggest that suboptimal CD4+ helper responses are unlikely to explain the study outcome in all cases. Moreover, such an effect may more likely impact the durability of efficacy, which may be partially addressed in long-term follow up of the study. Of note, other candidate regimens under evaluation, including priming with HIV DNA followed by boosting with recombinant Ad5 or pox vectors containing HIV-1 inserts, typically induce higher CD4+ T helper response rates, which has been a leading argument for advancing these regimens to larger scale trials.

Our exploratory studies to understand why the vaccine may have increased infection risk in the Step study did not provide major insights. Certainly, HIV-specific CD4+ T cells were elicited in 46% of cases prior to infection, and while this has been considered a desired effect, these may preferentially serve as susceptible target cells for HIV-1 infection, as has been reported in HIV-infected individuals (33). This possibility is supported by a previous study demonstrating enhanced SIV replication and disease progression in non-human primates receiving a varicella-zoster virus vaccine expressing SIV envelope (34). However, enhanced infection resulting from vaccine-induced SIV- or HIV-specific CD4+ T cells has not been observed in the numerous published nonhuman primate SIV vaccine studies including those involving adenovirus-based candidates or in other clinical HIV vaccine efficacy trials, but whether this possibility was considered or adequately addressed is unclear. Two findings perhaps require further evaluation. One, the Ad5-specific T cell response rates were lower in

the cases than the non-cases, suggesting that these cells may have trafficked to mucosal sites, a process known to occur in natural infection, and thus increased the number of susceptible CD4+ T cell targets for HIV. Additionally, circulating CCR5-expressing activated CD4+ T cells were more abundant in persons with high Ad5 titers when assessing cryopreserved PBMC, which may have increased target cell susceptibility to HIV-1 following exposure at mucosal sites. While detectable levels of these cells in blood were not increased in the vaccine arm in comparison to the placebo arm, it remains unclear what may occur in tissue compartments at the site of HIV-1 infection. To address this possibility, studies are planned to examine lower GI tissue and foreskin after immunization for enhanced T cell activation.

The Step study outcome highlights the enormous challenges that lie ahead in developing an efficacious HIV vaccine. One notable issue is defining the immunologic responses that can better predict vaccine efficacy. Although some leads have emerged from examining correlates of immune protection with other efficacious vaccines and with successful control of chronic HIV-1 infection, the immune profile that will provide the most valuable protective response against HIV infection remains an enigma. While the validated assays employed here are a substantial improvement over traditional assays, we recognize that these may be insufficient in identifying the immunologic properties that most closely associate with an immune correlate for protection against mucosal HIV infection. Some key analyses are pending, and at a minimum, measuring the ability of CD8+ T cells to proliferate and to suppress HIV-1 replication is an important next step. In addition, a more thorough understanding of the total effector responses through transcriptional microarray and proteomics also takes priority for available stored specimens. Clues may come from comprehensive analysis of the protective immune correlates in the live attenuated SIV vaccine model in rhesus macaques, but in future clinical vaccine trials we must adopt multiple exploratory studies to gather leads on the response patterns that will be useful to measure. Further analyses of immune responses as predictors of the HIV-1 infection risk are being pursued, including survival analyses those that use the time to HIV-1 infection, and it will be important to verify findings reported here as more cases accrue in the study.

Obviously, an efficacious HIV vaccine must afford protection against heterologous virus, and the enormous variability of HIV-1 creates a major hurdle in designing a vaccine that can induce a sufficiently broad response to permit recognition (35–37). This barrier may be one that ultimately compromises the effectiveness of T cell-based vaccines. Future analyses of the Step study samples will reveal the extent of coverage the MrkAd5/HIV-1 vaccine provided, which will guide improved vaccine designs. Strategies hold promise that improve T cell breadth of relevant epitopes using HIV-1 inserts that provide enhanced coverage of circulating strains, such as more centralized or even mosaic HIV inserts (38,39). Further, optimizing the functional antiviral responses of the T cells elicited, based on findings from more sophisticated genomics and proteomics approaches (40), may improve the chances for success in achieving long-term antiviral CD8+ T cell memory against HIV-1 infection. Faced with an epidemic that will be best halted by an effective vaccine, there is no better time to channel knowledge from data, not hindsight or opinion, into careful planning for the next steps in the search for a preventive HIV vaccine.

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Appendix

Author Contributions

MJM, ZM, SPB, MNR, DVM, SGS, LC, and JWS participated in the design of the study; MJM, SCD, and RA participated in the design, implementation and analysis of the flow cytometric experiments; LK participated in the Ad5 Ab assay development and implementation; SPB and MNR chaired the study protocol; MJM, SCD, SD, ODD, DKC, JH and DRC participated in the study implementation; MJM, JH, LC, JWS and DRC managed the study; SCD, ZM, HJ, ODD, DVM and SGS performed the data and statistical analysis; MJM, SCD, ZM, HJ, DVM, SGS, JWS, DC were involved with the data interpretation; MJM, SCD, ZM, HJ, LC and DRC participated in the writing of the manuscript.

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Figure 1.

Ex vivo HIV-specific CD4+ and CD8+ T cells induced by the Merck trivalent Ad5/HIV vaccine. A) Cytokine and activation marker flow cytometric staining profiles in previously cryopreserved PBMC from one vaccine recipient obtained four weeks after the third immunization (week 30). The Ad5 neutralizing titer for this individual is 893. Cells were gated by forward and side scatter, live vs. dead cells, CD3+ T cells, and then CD4+ and CD8+ T cells. HIV-specific CD4+ (left two columns) and CD8+ (right two columns) T cells were stimulated *ex vivo* with Gag, Pol and Nef peptide pools that span the sequence encoded by the HIV-1 gene insert. The numbers indicate the percent of CD4+ or CD8+ cells expressing cytokine(s) IFN- γ , IL-2 and/or TNF- α . The responses in the negative control were low (<0.01%

for all cytokines, not shown). B and C) The percent CD4⁺ (B) or CD8⁺ (C) T cells producing IFN- γ and/or IL-2 in response to HIV-1 Gag, Pol and/or Nef (left panels) or HIV-1 Gag only (right panels), four weeks after the second (week 8) or third (week 30) dose in vaccine recipients who remained HIV-uninfected (matched non-cases) or who became HIV-infected (cases). ICS analyses were performed on PBMC obtained prior to infection in cases. The participants are stratified by Ad5 neutralizing antibody titer (≤ 18 and >18). Indicated above each figure are the numbers of positive responders, both as a ratio and as a percentage. The red dots represent positive responders and the blue dots negative responders; triangles identify PBMC from week 8, and circles identify PBMC from week 30.

Figure 2.

Vaccine-induced HIV-specific CD4+ and CD8+ T cells producing multiple cytokines. The left graphs show the percentage of the HIV-specific CD4+ (A) or CD8+ (B) T cells that are producing 1, 2 or 3 cytokines in the vaccine recipients. Within each graph, cases are shown in blue and non-cases in red. ICS analyses were performed on PBMC obtained four weeks following the third vaccination (week 30). None of the cases analyzed were infected at this time point. The right graphs depict the percentage of cells producing IFN- γ , IL-2 or TNF- α among those producing one cytokine, and the percentage of cells co-producing cytokine pairs among those producing two cytokines.

Figure 3.

Ex vivo Ad5-specific CD4+ and CD8+ T cells. A) Ad5-specific CD4+ (left panels) and CD8+ T cells (right panels) in a representative donor (same as in Figure 1A). PBMC were stimulated overnight with empty Ad5 vector and evaluated (as in Figure 1A) for intracellular cytokine expression. B and C) The percent CD4+ (B) and CD8+ (C) T cells producing IFN- γ and/or IL-2 in response to Ad5 empty vector stimulation in vaccine recipient cases and matched non-cases. PBMC were analyzed from subjects obtained four weeks after the second (week 8) or the third (week 30) Ad5 vaccination. The participants are stratified by Ad5 neutralizing antibody titer (≤ 18 and > 18). The numbers of positive responders are indicated above each figure as a ratio and as a percentage. The red dots represent positive responders, and the blue dots signify the negative responders; triangles identify PBMC from week 8 and circles signify PBMC from week 30.

Figure 4.

Activated CD4⁺ T cells expressing CCR5. A) Activated T cells expressing CCR5 in a representative donor (same as in Figure 1A). The left panels depict the overall percentage of Ki-67⁺Bcl-2^{lo} activated CD4⁺ and CD8⁺ T cells, respectively. The right panels demonstrate the expression of CCR5 for the overall population of CD4⁺ or CD8⁺ T cells in grey, as well as the activated cells (red overlay). The numbers indicate the percent of activated CD4⁺ and CD8⁺ T cells expressing CCR5. B) Percent activated (Ki-67⁺/Bcl-2^{lo}) CD4⁺ T cells expressing CCR5 in vaccine recipient and placebo recipient non-cases and cases four weeks following the third Ad5 vaccination (week 30), stratified by Ad5 neutralizing antibody titer (≤200, red dots; >200, blue dots). Only cases infected after week 30 are included. C) Percent

activated CD4+ T cells expressing CCR5 in non-cases at six months following the third Ad5 vaccination (week 52), stratified by Ad5 neutralizing antibody titer.

Table 1

IFN- γ -secreting T cell responses in case and non-case vaccine recipients at week 8.*

Response Rate and Geometric Means (SFC/10 ⁶ PBMC)		Ad5 < 18		Ad5 > 18	
		Cases (n=15)	Non-cases (n=95)	Cases (n=23)	Non-cases (n=221)
HIV Antigens	% (CI)	GM % (CI)**	GM % (CI)**	% (CI)	% (CI)
Gag	73 (47, 90)	516 (75, 65, 82)	301 (57, 37, 74)	216 (60, 54, 66)	173
Pol	73 (47, 90)	875 (73, 63, 81)	541 (43, 26, 63)	335 (52, 46, 59)	260
Nef	73 (47, 90)	374 (67, 54, 76)	275 (57, 37, 74)	210 (57, 50, 63)	166
≥ 1 antigen	73 (47, 90)	-	83 (74, 89)	-	73 (67, 78)
≥ 2 antigens	73 (47, 90)	-	74 (64, 82)	-	57 (50, 63)
3 antigens	73 (47, 90)	-	58 (48, 67)	-	39 (33, 46)
HIV Antigens	Ad5 < 200				
		Cases (n=23)	Non-cases (n=143)	Cases (n=15)	Non-cases (n=173)
	% (CI)	GM % (CI)**	GM % (CI)**	% (CI)	% (CI)
Gag	70 (49, 85)	410 (76, 68, 82)	260 (53, 30, 75)	193 (54, 46, 61)	168
Pol	61 (41, 78)	686 (73, 66, 80)	463 (47, 25, 70)	292 (47, 40, 55)	241
Nef	70 (49, 85)	358 (70, 62, 77)	237 (53, 30, 75)	165 (51, 43, 58)	163
≥ 1 antigen	74 (53, 88)	-	86 (79, 91)	-	68 (61, 75)
≥ 2 antigens	65 (45, 81)	-	75 (67, 81)	-	51 (43, 58)
3 antigens	61 (41, 78)	-	58 (50, 66)	-	34 (27, 41)

* Data represent positive responses, defined as ≥ 55 SFC/10⁶ PBMC and ≥ 4 -fold over negative control in the IFN- γ ELISpot assay, using previously cryopreserved PBMC from vaccine recipients (random sample of ~25%) collected at week 8, 4 weeks after the second immunization. Only cases from the PP analysis are included; subjects with evidence of HIV infection before or at week 12 were excluded from the evaluation, and the cases analyzed here were diagnosed after week 12. Subjects are stratified by baseline Ad5 neutralizing Ab titer: ≤ 18 , >18 , ≤ 200 , and >200 . The geometric mean values are those from the spot-forming frequencies obtained from the HIV-1 peptide pools.

** CI, 95% confidence intervals (18)

Abbreviations: GM, geometric means (SFC/10⁶ PBMC)

Table 2
HIV-specific CD4+ and CD8+ T cell response frequencies in male vaccine recipients.*

HIV Antigens CD4+ T Cells	% Responders (95% Confidence Intervals)					
	Ad5 < 18			Ad5 > 18		
	Cases n=10	Non-cases n=52	Total	Cases n=16	Non-cases n=64	Total
Gag	50 (24, 76)	42 (30, 56)	44 (23, 67)	33 (23, 45)	46 (29, 65)	37 (29, 46)
Pol	30 (11, 60)	19 (11, 32)	6 (1, 28)	11 (5, 21)	15 (6, 36)	15 (9, 22)
Nef	0 (0, 28)	14 (7, 25)	13 (4, 36)	9 (4, 19)	8 (2, 24)	11 (7, 18)
Total	50 (24, 76)	44 (32, 58)	44 (23, 67)	36 (25, 48)	46 (29, 65)	40 (31, 49)
CD8+ T Cells	n=13	n=59	n=17	n=71	n=30	n=130
Gag	54 (29, 77)	61 (48, 72)	29 (13, 53)	25 (17, 37)	40 (25, 58)	42 (33, 50)
Pol	85 (58, 96)	80 (68, 88)	47 (26, 69)	37 (26, 48)	63 (46, 78)	56 (48, 64)
Nef	62 (36, 82)	70 (57, 80)	35 (17, 59)	44 (33, 55)	47 (30, 64)	55 (47, 64)
Total	100 (77, 100)	90 (80, 95)	53 (31, 74)	59 (48, 70)	73 (56, 86)	73 (65, 80)
T cells (CD4+ and/or CD8+)	n=13	n=60	n=18	n=73	n=31	n=133
Gag	62 (36, 82)	68 (56, 79)	50 (29, 71)	44 (33, 55)	55 (38, 71)	55 (46, 63)
Pol	92 (67, 99)	78 (66, 87)	44 (25, 66)	40 (29, 51)	65 (47, 79)	57 (49, 65)
Nef	62 (36, 82)	68 (56, 79)	33 (16, 56)	45 (34, 57)	45 (29, 62)	56 (47, 64)
Total	100 (77, 100)	88 (78, 94)	61 (39, 80)	66 (54, 76)	77 (60, 87)	76 (68, 82)

* Multiparameter flow cytometry was used to detect HIV-specific CD4+ or CD8+ T cells that express intracellularly IFN- γ , IL-2 or both cytokines. Data are shown for evaluable responses to each antigen, following *ex vivo* stimulation with the relevant peptide pools. Refer to Figure 1 and methods for additional details.