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Safety and immunogenicity of recombinant poxvirus HIV-1 vaccines in young adults on highly active antiretroviral therapy

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Abstract

A trial to evaluate the safety and immunogenicity of recombinant modified vaccinia Ankara (MVA) and fowlpox (FP) vectors expressing multiple HIV-1 proteins was conducted in twenty HIV-1 infected youth with suppressed viral replication on HAART. The MVA and FP-based multigene HIV-1 vaccines were safe and well tolerated. Increased frequencies of HIV-1 specific CD4+ proliferative responses and cytokine secreting cells were detected following immunization. Increased frequencies and breadth of HIV-1 specific CD8+ T cell responses were also detected. Plasma HIV-1-specific antibody levels and neutralizing activity were unchanged following vaccination. Poxvirus based vaccines may merit further study in therapeutic vaccine protocols.

Keywords

HIV-1; Vaccine; Therapeutic Immunization

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1. Introduction

Several lines of evidence suggest that HIV-1 specific CD4 and CD8 T-cell responses are important for controlling viral replication. Live viral vaccines generally tend to elicit stronger and more durable immune responses than inactivated virus or recombinant protein vaccines. Recombinant poxvirus vaccines are worthy candidates given the wealth of experience using vaccinia for smallpox immunization programs in humans, and the development of recombinant techniques that enable insertion of substantial lengths of foreign DNA [1–7]. More recently, attenuated strains of vaccinia have been developed (modified vaccinia Ankara; MVA) that are safer for use in young or immunocompromised hosts [6,8–10] while retaining their immunogenicity. Poxvirus vaccines, including MVA and fowlpox vaccines, have been shown to effectively elicit T-cell responses [11–13].

The present report describes a Phase I safety and immunogenicity trial of recombinant MVA and fowlpox vaccines administered to HIV-infected young adults on highly active antiretroviral therapy (HAART).

2. Materials and methods

This was a prospective, multicenter trial carried out by the Pediatric AIDS Clinical Trials Group (PACTG) and the International Maternal, Pediatric, Adolescent, AIDS Clinical Trials (IMPAACT) network. The study received IRB approval at all enrolling institutions and each participant provided written, informed consent.

2.1. Study subjects

The study enrolled HIV-1-infected individuals, ages 18–24 years, receiving stable HAART (≥ 3 agents from ≥ 2 drug classes) between October 17, 2005 and June 26, 2006. Inclusion criteria included: plasma HIV RNA levels (plasma viral load) < 100 copies/mL for at least 6 months prior to study enrollment; CD4⁺ cell counts > 350 cells/mm³; and clinically healthy with all laboratory values grade < 2 (baseline characteristics outlined in Table 1). Based on rare reports of myopericarditis following administration of a less attenuated strain of vaccinia in a prior study [14], individuals were excluded if they had a history of cardiac disease, electrocardiogram (ECG) abnormalities, or more than two cardiac risk factors. Finally, individuals were also excluded if treated with immunomodulating agents or previously immunized with a poxvirus or HIV vaccine.

2.2. Vaccine

The HIV-1 genes expressed by the candidate vaccines were derived from an HIV-1 isolate from a vertically HIV-1-infected infant designated as C58A1 (subtype B). The *env* gene contained the immunodominant portion of gp41. The *tat*, *rev*, *nef* and *reverse transcriptase* (*RT*) genes were modified to render the proteins non-functional. These genes were inserted into two MVA and two FPV vectors; one of each pair containing *env* and *gag*, and the other containing *tat*, *rev*, and a *nef-RT* fusion gene.

At study entry (week 0) and week 4, the two MVA-based vectors were administered as a set intramuscularly (deltoid) at doses of 5×10^7 pfu each. At weeks 8 and 24 the two FPV-based vectors were administered as a set intramuscularly at doses of 5×10^8 pfu each.

Clinical monitoring for symptoms took place for one hour, and symptom diaries were kept for one week following each immunization. All toxicities noted during the study were classified by the DAIDS Toxicity Table for Grading Severity of Adult Adverse Experiences®, dated August, 1992, and a protocol specific Supplemental Toxicity Table for reactogenicity occurring within 14 days post-vaccination.

Monitoring for cardiotoxicity involved ECGs and troponin I levels measured at entry and 2 weeks after each MVA vaccine dose. If abnormal at any visit, or if any subject developed symptoms or findings consistent with the new onset of myopericarditis, a standardized, protocol-defined, cardiac evaluation was completed.

2.3. Clinical laboratory evaluations

Complete blood counts, blood chemistries and tests to detect myopericarditis were performed in clinical laboratories at each study site. CD4 and CD8 T-cell counts, and the activation states of CD8 T-cells (HLA-DR and CD38 expression), were measured in a single laboratory using standard flow cytometry protocols (Tri-test; BD Pharmingen, San Jose, CA). Plasma viral loads (PVL) were measured using a RT-PCR assay (Roche Ultrasensitive Amplicor assay, version 1.50).

2.4. Immunogenicity studies

Whole blood samples were shipped overnight and were processed for assays or cryopreservation of blood components within 28 hours of phlebotomy. Lymphocyte proliferative responses to whole protein antigens were measured using a membrane dye dilution methodology adapted from da Silva et al [15]. PBMC were treated with 8 μ M PKH26, a fluorescent membrane dye, using the manufacturer's protocol (Sigma Chemical Co.), then cultured in duplicate at 2×10^5 per well in 96-well plates with the following antigens and controls: 1) recombinant HIV-1 p24 at 5 μ g/mL and 2) matched control protein (Protein Sciences, Meriden, CT); 3) Aldrithiol-2-inactivated (AT2) HIV-1_{MN} at 300ng/mL of p24 and 4) matched microvesicle control (kindly provided by Dr. Jeffrey Lifson; AIDS Vaccine Program, National Cancer Institute, Frederick, MD); 5) Candida antigen at 50 μ g/mL (Greer Laboratories, Lenoir, North Carolina); and 6) complete medium. AT2 HIV-1_{MN} are inactivated viral particles that retain the ability to bind, fuse and enter CD4⁺ CCR5⁺ cells. They were included in this and other assays specifically to measure CD8 T-cell responses [16]. After a 6-day incubation, cells were harvested and stained with fluorochrome-conjugated antibodies against CD3 (APC), CD4 (FITC) and CD8 (CyChrome) (BD Pharmingen, San Jose, CA) for flow cytometry. Proliferation indices (PI) of CD4 and CD8 T-cells were determined using MODFIT software (Verity software house, Topsham, ME).

Cytokine responses (Interleukin-2 and Interferon- γ ; IL-2 and IFN γ) were measured by flow cytometry using standard methods (ACTG Laboratory Technologist Committee Manual). Whole HIV-related antigens included HIV-1 Gag (p55; Austral); HIV-1 Nef (produced at UMMS); and AT2 HIV-1_{MN}. Controls included control microvesicles; CMV surface glycoprotein, pp65; SEB; medium alone; and a pool of peptides that include known optimal epitopes of CMV, EBV and Influenza virus (CEF).

MVA epitope-specific CD8 T-cells were quantified using APC-labeled peptide-MHC-1 tetramer complexes with peptide epitopes identified by Terajima et al ([17]; 74a and 165). HLA-A*0201 positive individuals were identified by molecular haplotype assays (BioTest ABC SSPtray; BioTest Diagnostics Corp, Denville, NJ). Vaccinia-specific CD8 T-cell IFN γ and MIP-1 β production was by flow cytometry measured using stimulation with live vaccinia virus (NYCBH strain) at an MOI of 5 as described by Precopio et al [18].

ELISPOT assays were performed utilizing Mabtech™ ELISpot plus Human Interferon-gamma kits (MABTECH AB, Sweden). Cryopreserved PBMC from four selected time points (screen, entry, week 6 and week 26) for each individual were tested together in a single assay to avoid interassay variability. Clade B consensus peptides were obtained from the NIAID Reagents Program (<https://www.aidsreagent.org/index.cfm>) and were pooled as described in Table 2. Positive controls included SEB, PHA, and CEF. Negative controls included wells with medium

and cells but no stimulus. This background was subtracted from results of wells with peptides in the analyses. Positive responses were defined for each individual as $\geq \text{mean} + 3 \times \text{standard deviations (SD)}$ for control wells and a minimum of 3 spots/well.

Polychromatic flow cytometry was used to further characterize the functional properties of T-cell responses in selected individuals with prominent responses to peptide pools representing highly conserved regions of the HIV genome (Gag and Pol). PBMC were stimulated with selected pools of OLP, and live vaccinia virus as described [18]. Anti-CD107 (FITC-conjugated) was added with peptide in the presence of Brefeldin A and monensin (BD Pharmingen). Following a 5-hour incubation, cells were fixed and incubated with a mixture of antibodies specific for: CD14/19 (Alexa700); CD3 (APC-Cy7); CD8 (PerCP); IFN γ (Pacific Blue); MIP-1 β (PE); TNF α (PE-Cy7); and IL-2 (APC); and the vital stain, Live/Dead Blue (Invitrogen). All antibodies were obtained in the conjugated form from BD Pharmingen with the exception of anti-IFN γ (Pacific Blue; E-bioscience). Flow cytometry was performed using a LSRII instrument (BD Bioscience, San Jose, CA) and data were analyzed using FlowJo software (TreeStar, Inc; Palo Alto, CA). Assays in which there were more than 500 reactive cells per million PBMC detected above background were analyzed using SPICE software (version 4.1.5) for presentation of patterns of functionality.

Sera were tested in batches using ELISA to detect antibodies that bound to viral lysates (HIV-1 Microelisa System; Vironostika™, Biomerieux, Durham, NC). Appropriate negative controls defined the threshold of detection in each assay ($\text{mean} + 3 \times \text{SD}$). Serial dilutions defined the antibody titer as the inverse of the dilution at which the threshold was crossed.

Serum neutralizing activity was measured using standardized assays adapted from previously published protocols [19,20]. Assays included env-pseudotyped reference viruses representative of B or C-clade variants, (NIH AIDS Reagents & Reference Reagent Program; <https://www.aidsreagent.org/index.cfm>), and the vaccine variant C58A1.7; kindly provided by David Montefiori).

2.5. Statistics

Assessment of study vaccine regimen safety was performed as an intent-to-treat analysis. Primary safety endpoints included: all grade ≥ 3 adverse events; grade ≥ 3 adverse events attributable to the study vaccine; viral breakthrough, defined as a confirmed PVL > 1000 copies/mL during the first 26 weeks, not explained by poor adherence; or CD4 cell decline by at least 5%, to $< 20\%$.

Baseline and post-immunization immune responses were compared using the Wilcoxon sign rank test. Baseline values comprised the average of screen and entry results. The individual who received only one MVA immunization was excluded from all analyses.

Thresholds to define positive proliferation and cytokine responses measured by flow cytometry were calculated as the mean $+ 2 \times \text{SD}$ of control assays without antigen stimulation over all subjects. Thresholds to define positive ELISPOT responses were calculated as the mean $+ 3 \times \text{SD}$ of control assays without antigen stimulation for each batch of samples on an individual subject.

3. Results

3.1. Enrollment and vaccination

Baseline characteristics of study participants are summarized in Table 1. At baseline, all 20 participants had PVL < 50 copies/mL, and the median CD4 $^{+}$ cell count was 815 cells/mm 3 (range 389 – 1956 cells/mm 3). Median age was 23 yrs (range 19–24). Eleven participants were

male and 9 were female; 11 were Black (not Hispanic), 7 were Hispanic, and 2 were White (not Hispanic). Five (25%) individuals had acquired HIV-1 perinatally and 15 (75%) were infected through sexual transmission.

Eleven of the participants received all 4 sets of scheduled immunizations (MVA at 0 and 4 weeks, FP at 8 and 24 weeks; Fig. 1). Seven additional participants received 3 sets of immunizations (MVA at 0 and 4 weeks, FP at 8 weeks). Six of these 7 individuals did not receive the final set of FP immunizations at 24 weeks due to closure of the vaccine manufacturer, Therion Biologics Corporation, for financial reasons. One of these 7 individuals discontinued HAART just prior to the 24-week study visit and did not receive the final set of FP immunizations. Finally, immunizations were discontinued after 1 set of immunizations in one individual (due to a grade 3 rash “probably not” due to study treatment), and after 2 sets of immunizations in another individual who developed elevated CPK levels.

3.2. Adverse events

Eight grade ≥ 3 adverse events were noted. Six of these adverse events were not considered to be treatment-related but were believed to be associated with underlying HIV disease, antiretroviral therapy, or incidental trauma. Two events were considered possibly but probably not related to the vaccines. One subject had a papular lesion present at the time of immunization with subsequent development of multiple cutaneous bullous lesions; none at the site of immunization. Culture of fluid grew group A *Streptococcus* and *Staphylococcus aureus*, so a concurrent and unrelated episode of impetigo was diagnosed. However, a skin biopsy was reported as “possibly consistent” with a drug eruption, so the final attribution was “probably not” related. A second subject developed grade=4 elevation of CPK at week 6. An alternative cause could not be identified, however it was noted that unexplained CPK elevations had occurred prior to study participation (Fig. 1).

Mild (and occasionally moderate) pain and tenderness at the injection site was common, but resolved within 8 days. Despite aggressive scrutiny for cardiotoxicities, none were identified.

3.3. CD4 and CD8 T-cell counts and activation status

CD4 and CD8 T-cell percentages and absolute counts remained stable over the first 48 weeks, as did absolute counts of activated CD8 T-cells (data not shown). No subject met the predefined study endpoint for CD4 decline.

3.4. Plasma HIV-1 RNA levels

Fifteen (75%) of the study participants maintained PVL < 50 copies/ml through week 24 of study (Fig. 1). Three subjects had transient (single time point), low level viremia (59–497 copies/mL) detected during the first 24 weeks on study. Two individuals had PVL increases to > 1000 copies/ml at week 24 due to discontinuation of HAART.

Five subjects had detectable viremia at week 60 and/or 72 (64–175 copies/mL); 3 returned to < 50 copies/ml at week 72. One subject (Subject 11) experienced a consistent rise in viremia despite reportedly good adherence to his antiretroviral regimen (53, 6,319, and 87,121 copies/mL; at weeks 48, 60 and 72, respectively; Fig. 1). Breakthrough viremia was with archival drug-resistant virus detected at baseline (Shiu et al.; manuscript in preparation).

3.5. Vaccinia-specific CD8 T-cells

All study participants were vaccinia naïve, and vaccinia-specific CD8 T cells were not detected at baseline. Vaccinia-specific CD8 T cell cytokine responses were evaluated in the 19 individuals who had received both MVA immunizations. Samples obtained between study weeks 24 and 32 (i.e., 20 to 24 weeks after the second MVA immunization) were used for

testing responses of all but 2 individuals whose week 6 samples were used. Vaccinia-specific CD8 T cell responses were detected in 14 (74%) of the 19 individuals.

Using four-color flow cytometry to detect IFN γ and MIP-1 β responses, background-subtracted vaccinia-specific CD8 T cell frequencies ranged from 0 to 0.078% (median 0.02%). Polychromatic flow cytometry demonstrated polyfunctional responses and significant increases in frequencies of CD8 T cells responding to live vaccinia stimulation (representative and aggregate data shown in Figs. 2 A–C). One individual (subject 9; Figs. 2 A & B) had sufficient numbers of vaccinia-specific CD8 T cells to quantify each combination of cytokine responses. Thirty eight percent of vaccinia-specific CD8+ T cells demonstrated 3 or more functions following antigen-specific stimulation. The majority (70%) of vaccinia-specific CD8 + T cells expressed CD107 (a marker for degranulation), or secreted MIP-1 β (80%) following stimulation. Thirty eight percent of vaccinia-specific CD8+ T cells secreted TNF- α , 28 percent expressed IL-2, and 36 percent expressed IFN γ .

Vaccinia epitope-specific CD8 T-cells were also detected directly *ex vivo* by tetramer staining at week 6 in two of eight HLA-A*02 positive participants ($\geq 0.05\%$ above baseline; data not shown).

3.6. Plasma HIV-1 specific antibodies and neutralizing activity

HIV-1 specific antibody titers were high at baseline and did not change significantly among individuals who received all immunizations and who remained on HAART. Plasma neutralizing activity also did not change significantly (baseline data are shown in Table 1).

3.7. HIV-specific cellular immune responses

3.7.1 Proliferation—HIV-1 specific CD4 T cell proliferative responses were detected in 3 (15%) of the 20 participants at baseline, and in 8 (42%) of the 19 participants at 6 weeks (Fig. 3A). Significant increases in CD4 T cell proliferation indices (PI) to p24^{Gag} and adriathiol-2-inactivated HIV_{MN} (AT2 HIV) were detected at week 6 (n=19; p<0.001). These responses were sustained through weeks 26 and 48 in individuals who received all four immunizations and remained on HAART (n=10; p<0.05; Fig. 3B).

It appeared that p24^{Gag}-specific CD4 T cell proliferation at week 26 was greater among the 10 participants who received all immunizations when compared to the more inclusive group of 19 (which included 7 individuals who did not receive the final FP immunization and 1 who did not receive either FP immunization). This inclusive group also included two individuals who were no longer receiving ARV at the week 26 time point (one who received 3 immunizations and one who received all 4 immunizations). Thus, 15 individuals remained on HAART and received at least three immunizations. The mean p24^{Gag}-specific CD4 T cell proliferation at week 26 for the 10 who received 4 immunizations compared with the 5 who received 3 immunizations were 0.3975 and 0.1006, respectively (Mann-Whitney U Test; p<0.05).

CD8 T-cell proliferative responses to AT2 HIV were detected at baseline in 9 of 20 individuals (Table 1). Significant increases in CD8 T-cell proliferative responses were not observed following immunization (data not shown).

3.7.2 Gag-specific CD4+ T cell cytokine production to whole antigens—Gag-specific IL-2 secreting CD4+ T cells were detected in 4 of 20 (20%) individuals at baseline (Table 1), and in 12 of 19 (63%) individuals at week 6. Increased frequencies of Gag-specific interleukin-2-secreting CD4 T-cells were observed at week 6 (Fig. 3C; p=0.003) but did not

persist at later time points. Similar, but non-significant trends were observed for gag-specific IFN γ -producing CD4 T-cells (Fig. 3D).

3.7.3 Cytokine responses to peptide pools—At baseline, IFN γ responses to one or more HIV-1 peptide pools were detected by ELISPOT in all individuals (Table 2; Fig. 4; range 1 to 10; median = 6 pools recognized). At week 6, 12 of 19 (63%) individuals had an increase in ELISPOT responses to ≥ 1 additional peptide pools. At week 26, 9 of 10 (90%) of individuals had an increase in ELISPOT responses to ≥ 1 additional peptide pools. Responses to a significantly greater number of peptide pools (median increase = 2) were observed at weeks 6 and 26 ($p=0.03$ and 0.04 respectively; Fig. 4A). In aggregate, significant increases in frequencies of SFC were observed in response to two pools of Pol peptides (including amino acids 178–556 of reverse transcriptase), and one pool of Gag peptides spanning amino acids 245–500 (Table 2). Low-level increases in frequencies of responses to Tat and Rev peptides, and non-significant positive changes from baseline additional pools of Gag and Nef were also observed (Table 2). Responses to Env peptide pools remained unchanged. Summed frequencies of responses to OLP (excluding Env OLP) were significantly increased at weeks 6 ($n=19$) and 26 ($n=10$) (Fig. 4B).

CD8 T-cell responses were characterized in greater detail in selected participants ($n=5$) with increased Gag or Pol-specific responses. HIV-1 specific CD8 T-cells produced fewer cytokines or degranulation markers than vaccinia-specific CD8 T-cells (Fig. 2C), consistent with previous reports [18]. In three individuals, increases in frequencies of HIV-specific CD8 T-cells were associated with improved functionality (examples shown in Figs. 5 A–B). In other individuals, increased HIV-1 specific CD8+ T cell frequencies were accompanied by a stable functional profile (Figs. 5 C–D).

4. Discussion

The candidate MVA and FP HIV-1 vaccines were well tolerated in HIV-infected young adults on HAART. Prior trials of MVA vaccines in HIV-1 infected individuals have also demonstrated favorable safety profiles [1,9,21]. While these trials used different products and doses, the collective experience suggests that MVA and FP vaccines can be safely used in HIV positive individuals.

Based on recent reports linking attenuated vaccinia immunizations with cardiac toxicities in healthy volunteers, study participants were carefully monitored for potential cardiac toxicity. The MVA used for this vaccine is further attenuated, and in spite of intensive monitoring, we found no significant cardiac events among study participants. Given the large numbers of normal variants that are often reported as abnormalities, the level of surveillance employed in this study was very labor intensive and not practical or feasible for larger studies.

Immune correlates of protection from HIV-1 disease progression are incompletely defined, however cellular immune responses are considered critical in limiting viral replication. HIV-1-specific CD4 T-cell proliferative responses have been correlated inversely with PVL and disease progression [22,23]. CD4 T-cells that produce both IFN and IL-2 in response to peptide stimuli also have an inverse relationship with PVL [24]. We observed significant augmentation of proliferative and IL-2 producing HIV-1 specific CD4 T-cell responses after immunizations. The enhancement of proliferative responses, in particular, appeared to be quite durable providing evidence that these vaccines may provide some long-term benefit.

The frequencies and functional profiles of vaccinia-specific CD8 T-cell responses were similar to those of healthy MVA recipients [18]. Augmentation of HIV-1 specific CD4 and CD8 T-cell responses in the context of new responses to poxvirus epitopes suggests specific boosting

by the vaccines. The candidate vaccines elicited a different hierarchy of responses (Gag>Pol>Nef>Env) in this trial than observed in a trial of the same vaccine regimen in HIV-1 uninfected adults (Env=Gag>Pol>Nef) [25]. This suggests that vaccination of the HIV-1 positive individuals may have resulted in the expansion of CD8 T-cells that were previously primed by natural infection. The larger magnitude of the HIV-1 specific responses compared with vaccinia-specific responses also suggests prior priming. The increased breadth of detectable HIV-1 specific CD8+ T cell responses (Fig. 4) may represent de novo responses or amplification of pre-existing low-level responses.

While the magnitude of vaccine-specific CD4 and CD8 T cell responses is likely to relate to vaccine effectiveness, functional properties of vaccine-elicited responses may also be important. In our studies, significant increases in CD4 T cell proliferation and CD8 T cell IFN γ production were detected. Direct comparisons of the frequencies and magnitude of vaccine-elicited responses are difficult due to differences in methodologies amongst therapeutic immunization trials. Nevertheless, the increases in response frequencies, as well as in the magnitude of responses, are similar to those previously reported from several trials. For example, the CD8 T cell IFN γ responses in the current study are of the same order of magnitude as those reported from a study of similar design [26]. However, there are no clear quantitative thresholds that define effective responses at this time. Future therapeutic vaccine studies that incorporate a period of HAART discontinuation following immunization could help to us to better understand whether the increased frequencies, or particular functional properties of vaccine-elicited responses are associated with improved control of viral replication.

It is generally accepted that potent (highly “functional”) responses against conserved viral epitopes with functional constraints on mutability would be most effective at controlling viral replication. HIV-specific CD8+ T-cells detected in infected individuals tend to produce fewer cytokines in response to their cognate antigens than those specific for antigens of other viruses [18]. It is therefore of note that we observed increased functionality of some HIV-1 epitope-specific CD8+ T-cells in several individuals after immunization.

In a recent trial of live recombinant adenoviral HIV vaccinations, increased susceptibility to infection was observed in individuals with pre-existing adenoviral immunity. In this context, our results and those of others suggest that future development of poxvirus based vaccines may deserve greater attention [27,28]. Broad segments of the population at risk for HIV infection lack pre-existing immunity to poxviruses, so are more likely to respond and perhaps less likely to display enhancement of infection.

In summary, this and other studies demonstrate the safety and immunogenicity of recombinant poxviruses, even in HIV-infected individuals [1,26,29]. While this particular recombinant vaccine is not likely to move forward in development, therapeutic trials of other poxvirus-based vaccines have been proposed or are in progress for HIV. Published results from early trials indicate that poxvirus-based vaccines are safe and immunogenic in adults with pre-existing immunity to tuberculosis and malaria [30,31]. Our results demonstrating broad boosting of CD4 and CD8 T-cell responses following vaccination are consistent with data from prior studies and support further evaluation of poxvirus-based vaccines.

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Fig. 1. Flow diagram of enrollment, immunization, viral loads and antiretroviral therapy

*Viremia detected: HIV-1 RNA copies/ml (c/ml) plasma (weeks when detected); ARV – antiretrovirals. ** Viremia detected at early time points ($\leq$ week 26) highlighted in underlined italics. *** Subject 11 had progressive increases in viremia on ARV; the three time points with viremia detected are listed

Fig. 2. Detection and enumeration of vaccinia-specific CD8 T-cells

A. The CD8 T-cell responses to live vaccinia stimulation (subject 9); dot plot. Top panels display cytokine and degranulation responses to vaccinia at study entry compared with background values. Bottom panels display these responses at study week 26, after all four immunizations.

B. The CD8 T-cell responses to live vaccinia stimulation (subject 9); functional profile. Depicted values are background subtracted using the no antigen control assay. Pie chart depicts the percentages of responding CD8 T-cells that express these markers individually and in combinations up to 5. Bar chart depicts the percentages of responding CD8 T-cells that express each of the possible combinations of markers (dots indicate positive responses).

C. Summation of CD8 T-cell cytokine responses to live vaccinia stimulation. Frequencies of responses at baseline and post-immunization among 5 individuals studied by this technique. Median, interquartile range and SD are shown by heavy line, bar and whiskers, respectively. Background subtracted values less than the threshold defined by the 90th percentile of a normal distribution around zero were assigned a value of 0 for calculation purposes. Wilcoxon sign rank test comparing post-immunization with baseline frequencies.

Fig. 3. HIV-specific CD4 T-cell responses

A. Percentage of individuals with positive CD4 T-cell proliferation responses to stimulation with p24^{Gag} at time points pre and post immunizations. Two groups shown are those who received all immunizations (n=10) and those who received at least two of the immunizations (n=19; including two who discontinued ARV before week 26).

B. HIV-specific CD4 T-cell proliferation. Proliferation index (PI) in response to p24^{Gag} are shown at baseline and week 6 for all participants who received at least two immunizations; and shown at week 26 and 48 are only those who received all 4 immunizations (one assay at week 48 was not performed due to sample handling problems).

C. HIV-specific CD4 T-cell cytokine responses; IL-2 producing, p55^{Gag}-specific CD4 T-cells.

D. HIV-specific CD4 T-cell cytokine responses; IFN γ producing, p55^{Gag}-specific CD4 T-cells. Panels C and D show baseline and week 6 values for all participants who received at least two immunizations; and values at week 26 for those who received all 4 immunizations. In panels B, C, and D, Wilcoxon sign rank test p-values shown compare baseline with week 6 (n=19); baseline with week 26 (n=10), and baseline with week 48 (n=9).

Fig. 4. HIV-specific CD8 T-cell responses

A. Overlapping pools of peptides were used to detect HIV-1-specific, IFN γ producing cells in an ELISPOT assay. PBMC were stimulated with pools as listed in Table 2. Wilcoxon sign rank test p-values shown compare baseline with week 6 (n=19), and baseline with week 26 (n=10).

B. Sum of IFN γ producing cells in response to OLP (sum of Gag, Nef, Pol, Rev, Tat responses; background subtracted) as measured by ELISPOT. Baseline is average of screen and entry values. Median values are indicated with a horizontal bar. Wilcoxon sign rank test p-values shown compare baseline with week 6 (n=19), and baseline with week 26 (n=10).

Fig. 5. HIV-specific CD8 T-cell responses

Participants with pronounced increases in IFN γ producing cells to pools of Gag and Pol OLP were selected to study the production of additional cytokines and degranulation markers in response to the same peptide pools.

A. Functional profile of CD8 T-cells responses to Gag Pool 1 at week 26 (subject 9; see also vaccinia responses; Fig. 2B). Frequencies of Gag Pool 1 CD8 T-cell responses at entry were insufficient to represent data in this format.

B. Functional profile of CD8 T-cells responses to Pol Pool 1 at baseline, week 6 and week 26 (subject 2; note that week 26 profile possibly influenced by episode of viremia at week 24). Frequencies (percentages) of Pol Pool 1-specific CD8 T-cells: entry = 0.18; week 6 = 0.15; week 26 = 0.68.

C. Functional profile of CD8 T-cells responses to Gag Pool 1 at baseline, week 6 and week 26 (subject 2; note that week 26 profile possibly influenced by episode of viremia at week 24). Frequencies (percentages) of Gag Pool 1-specific CD8 T-cells: entry = 0.13; week 6 = 0.09; week 26 = 0.15.

D. Functional profile of CD8 T-cells responses to Pol Pool 1 at baseline and week 26 (subject 11). Frequencies (percentages) of Pol Pool 1-specific CD8 T-cells: entry = 0.65; week 26 = 1.03.

Table 1

Baseline Characteristics of the 20 Study Participants

Characteristic	Number	Percent
Male	11	55
White, non-Hispanic	2	10
Black, non-Hispanic	11	55
Hispanic (regardless of race)	7	35
Median	Range	
Age (years)	23	19–24
CD4+ lymphocytes (%)	38	18–51
CD4+ lymphocytes (cells/mm ³)	815	389–1956
CD8+ lymphocytes (%)	42	30–67
CD8+ lymphocytes (cells/mm ³)	897	414–2111
Median	Range	
CD4 T cell proliferation to p24 ^{Gag}	0.046*	0–1.12(# positive: 3/20)
CD8 T cell proliferation to AT2 HIV	0.206	0.001–3.798 (# positive: 9/20)
CD4 T cell IL-2 production to p55 ^{Gag}	0.03 %	0–0.1% (# positive: 4/20)
CD4 T cell IFN γ production to p55 ^{Gag}	0.04 %	0–0.13% (# positive: 10/20)
CD8 T cell IL-2 production to AT2 HIV	0%	0–0.03% (# positive: 0/19**)
CD8 T cell IFN γ production to AT2 HIV	0.02%	0–0.83% (# positive: 2/19**)
Neutralizing activity against vaccine Env	307***	52–3,299

* Proliferation index as calculated by Modfit (“the sum of the cells in all generations divided by the computed number of original parent cells theoretically present at the start of the experiment”) with background subtracted. Other frequencies of virus-specific T cells are also background subtracted.

** One result excluded due to high background values.

*** Reciprocal dilution; IC₅₀

Table 2

HIV-1 Specific IFN γ Responses Measured by ELISPOT

Peptide (Pool #) # of peptides (amino acids)	Baseline Frequencies*	Change from Baseline** Week 6 (Post MVA) n=19	Change from Baseline** Week 26 (Post FP) n=10
Gag (1) 61 (1–255)	188 (70, 500)	92 (–57, 258) p = 0.258	281 (–103, 494) p = 0.105
Gag (2) 62 (245–500)	86 (34, 249)	64 (–7, 131) p = 0.130	60 (9, 455) p = 0.020
Pol (1) 83 (1–343)	39 (2, 296)	16 (–2, 126) p = 0.167	36 (–51, 366) p = 0.322
Pol (2) 83 (333–675)	24 (14, 256)	43 (18, 152) p = 0.010	80 (37, 146) p = 0.004
Pol (3) 83 (665–1003)	41 (20, 79)	19 (–9, 104) p = 0.031	39 (15, 179) p = 0.008
Env (1) 71 (1–295)	33 (3, 75)	0 (–35, 38) p = 0.644	–3 (–8, 12) p = 0.910
Env (2) 71 (285–579)	7 (0, 82)	2 (–12, 56) p = 0.495	0 (–5, 6) p = 0.938
Env (3) 70 (569–857)	87 (18, 186)	–8 (–42, 56) p = 0.899	–3 (–83, 7) p = 0.820
Tat 23 (1–101)	0 (0, 21)	8 (0, 35) p = 0.019	3 (0, 33) p = 0.078
Rev	1 (0, 20)	5 (–4, 63)	25 (3, 57)

Peptide (Pool #) # of peptides (amino acids)	Baseline Frequencies*	Change from Baseline** Week 6 (Post MVA) n=19	Change from Baseline** Week 26 (Post FP) n=10
27 (1-117)		p = 0.102	p = 0.014
Nef	196 (152, 405)	54 (-56, 521)	29 (-319, 1050)
49 (1-206)		p = 0.123	p = 0.193

* SFC/million PBMC; Median frequencies (interquartile range); values background subtracted.

** SFC/million PBMC; Median change from baseline, (interquartile range); p-values of Wilcoxon sign rank test comparisons of baseline with post-immunization responses, shaded cells indicate p <0.05.