STATISTICAL ANALYSIS PLAN FOR HVTN IMMUNOGENICITY

Protocol HVTN 139 (v1.0)

A phase 1 clinical trial to evaluate the safety and immunogenicity of HIV-1 vaccines based on chimpanzee serotypes of adenovirus expressing clade C gp140 and a CH505TF gp120 protein boost in healthy, HIV- uninfected adult participants.

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Statistical Analysis Plan for Immunogenicity

Protocol: HVTN 139 (1.0)

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Author:

Legal Name	Pei-Chun Yu, MSc
Job Title	Statistical Research Associate III
Signature & Date	See eTMF signature manifest

Approval:

Legal Name	Youyi Fong, PhD
Job Title	Professor
Signature & Date	See eTMF signature manifest

SAP Modification History

The version history of, and modifications to, this statistical analysis plan are described below.

SAP Version	Modification
1.0	Initial
2.0	Update Ad Nab, Appended ICS, Nab, BAMA specific details to section
3.0	Update ICS and BAMA
4.0	Update exclusion in section 8.1.1 general approach
5.0	Update Nab section to make it consistent with protocol secondary endpoints
6.0	Update ICS section per lab's feedback
7.0	Add analysis for ICS memory markers

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1 OVERVIEW

The following describes the Statistical Analysis Plan (SAP) for the analysis of immunogenicity data from HVTN 139 for Protocol Team (PT) reports for immunogenicity data, and the FSR for Immunogenicity.

2 PROTOCOL SUMMARY

Title

A phase 1 clinical trial to evaluate the safety and immunogenicity of HIV-1 vaccines based on chimpanzee serotypes of adenovirus expressing clade C gp140 and a CH505TF gp120 protein boost in healthy, HIV- uninfected adult participants.

Primary objective

To evaluate the safety and tolerability of AdC6-HIVgp140 and AdC7-HIVgp140 at doses of 1 x 10^{10} virus particles (vp) and 5 x 10^{10} vp, alone and in combination with CH505TF gp120 adjuvanted with GLA-SE in HIV- uninfected adults.

Study products and routes of administration

- **AdC6-HIVgp140**: a chimpanzee-derived replication-defective adenovirus (Ad) vector expressing codon optimized gp140 of clade C isolate Du422 administered at a dose of 1 x 10^{10} vp or 5 x 10^{10} vp. The vaccine dose will be divided equally into two separate 1mL intramuscular (IM) injections, administered into the deltoid of the non-dominant arm unless medically contraindicated.

- **AdC7-HIVgp140**: a chimpanzee-derived replication-defective Ad vector expressing codon optimized gp140 of clade C isolate Du172 administered at a dose of 1 x 10^{10} vp or 5 x 10^{10} vp. The vaccine dose will be divided equally into two separate 1mL IM injections administered into the deltoid of the non-dominant arm unless medically contraindicated.

- **CH505TF gp120**: CH505 transmitted/founder gp120 mixed with GLA-SE [the immunological adjuvant Glucopyranosyl Lipid A (GLA) in an oil-in-water stable emulsion (SE)], administered at a dose of 400 mcg with 10 mcg GLA-SE as a 1 mL injection in the thigh unless medically contraindicated.

- Placebo control for AdC6 -HIVgp140 and AdC7-HIVgp140 vaccine: Sodium Chloride for Injection, 0.9%, administered as two separate 1 mL IM injections into the deltoid of the non-dominant arm unless medically contraindicated.

- Placebo control for CH505TF gp120: Sodium Chloride for Injection, 0.9% administered as a 1 mL IM injection in the thigh unless medically contraindicated.

Participants

34 healthy, HIV– uninfected volunteers aged 18 through 50 years, inclusive; 30 vaccinees, 4 placebo recipients.

Schema

Table 2-1 Schema

Study arm	N	AdC dose (vp)	M0	M3	M6
Part A: Low Dose					
Group 1	5	1x 10^{10}	AdC6-HIVgp140	-	-
Group 2	5	1x 10^{10}	AdC7-HIVgp140	-	-
Group 3	2	0	Placebo	-	-
Part B: High Dose					
Group 4	10	5 x 10^{10}	AdC6-HIVgp140	AdC7-HIVgp140	400 mcg CH505TF gp120/GLA-SE
Group 5	10	5 x 10^{10}	AdC7-HIVgp140	AdC6-HIVgp140	400 mcg CH505TF gp120/GLA-SE
Group 6	2	0	Placebo	Placebo	Placebo
Total	34 (30 vaccinees / 4 placebos)				

Notes:

Study is blinded as to Group assignment (Part assignment is not blinded). Enrollment will be stepwise starting with Part A. To ensure the safety of participants, a series of pre-planned enrollment pauses will occur.

Safety Review #1: Low-dose Initial Vaccination. Enrollment for Part A will be restricted to a maximum of 1 participant per day across all participating HVTN Clinical Research Sites (CRSs) until a total of 5 participants have been enrolled. The HVTN Protocol Safety Review Team (PSRT) will review available safety and reactogenicity data reported for each of these 5 participants, up to and including the data reported for the first 72 hours postvaccination for the 5th enrolled participant, and determine whether it is safe to proceed with full enrollment in Part A.

Safety Review #2: Low-dose (Part A) Safe-to-Proceed. The HVTN PSRT will review cumulative safety data, including at a minimum the 2-weeks following the vaccination visit, available on all 12 participants in Part A to determine whether dose-escalation may occur from Part A to Part B.

Safety Review #3: Full Enrollment at Targeted Dose. The HVTN PSRT will also review cumulative safety data available on the first 12 participants enrolled in Part B, including the 2-week post Month 0 vaccination visit, to determine whether it is safe to proceed with full enrollment.

Design

Multicenter, randomized, controlled, double-blind trial.

Safety monitoring

HVTN139 PSRT; HVTN Safety Monitoring Board (SMB)

3 OBJECTIVES AND ENDPOINTS

3.1 Primary

Primary objective 1:
To evaluate the safety and tolerability of AdC6-HIVgp140 and AdC7-HIVgp140 at doses of 1 x 10^{10} virus particles (vp) and 5 x 10^{10} vp, alone and in combination with CH505TF gp120 adjuvanted with GLA-SE in HIV- uninfected adults.

Primary endpoint 1:
Local and systemic reactogenicity signs and symptoms for a minimum of seven days following receipt of any study product. All adverse events for thirty days after any receipt of study vaccination. All serious adverse events (SAEs) medically attended adverse events (MAAEs), and adverse events of special interest (AESIs) will be collected for twelve months following any receipt of study product.

3.2 Secondary

Secondary objective 1:
To evaluate and compare immune responses elicited by single administration of AdC6-HIVgp140 or AdC7-HIVgp140 at two different dose levels.

Secondary endpoints 1:

- Response rate, magnitude, and breadth of HIV-specific serum IgG binding antibodies assessed 4 weeks after a single vaccination (Part A) or the first vaccination (Part B)
- Response rate, magnitude, and polyfunctionality of HIV-specific CD4+ and CD8+ T-cell responses assessed 4 weeks after a single vaccination (Part A) or the first vaccination (Part B)
- Response rate, magnitude, and breadth of serum neutralizing antibodies against tier 1 and if applicable, other heterologous tier 2 HIV-1 isolates assessed 4 weeks after a single vaccination (Part A) or the first vaccination (Part B)

Secondary objective 2:

- To evaluate and compare immune responses elicited by sequential administration of AdC7-HIVgp140 followed by AdC6-HIVgp140 and AdC7-HIVgp140 followed by a dose of AdC6-HIVgp140 at 5 x 10^{10} vp

Secondary endpoints 2:

- Response rate, magnitude, and breadth of HIV-specific serum IgG binding antibodies assessed 4 weeks after the second vaccination (Part B)
- Response rate, magnitude, and polyfunctionality of HIV-specific CD4+ and CD8+ T-cell responses assessed 4 weeks after the second vaccination (Part B)

- Response rate, magnitude, and breadth of serum neutralizing antibodies against tier 1 and if applicable, other heterologous tier 2 HIV-1 isolates assessed 4 weeks after the second vaccination (Part B)

Secondary objective 3:

To evaluate and compare immune responses elicited by heterologous AdC7-HIVgp140 followed by AdC6-HIVgp140 or AdC7-HIVgp140 followed by AdC6-HIVgp140 prime followed with CH505TF gp120 / GLA-SE boost.

Secondary endpoints 3:

- Response rate, magnitude, and breadth of HIV-specific serum IgG binding antibodies assessed 2 weeks after the third vaccination (Part B)
- Response rate, magnitude, and polyfunctionality of HIV-specific CD4+ and CD8+ T-cell responses assessed 2 weeks after the third vaccination (Part B)
- Response rate, magnitude, and breadth of serum neutralizing antibodies against tier 1 and if applicable, other heterologous tier 2 HIV-1 isolates assessed 2 weeks after the third vaccination (Part B)

3.3 Exploratory

Exploratory objective 1:
To characterize antibody avidity and/or Fc-mediated antibody functions (infected cells antibody binding assay [ICABA], antibody-dependent cellular cytotoxicity [ADCC], antibody dependent cellular phagocytosis [ADCP], antibody dependent neutrophil phagocytosis [ADNP], and FcR-binding)

Exploratory objective 2:

To assess the impacts of vaccination on B-cell subset distribution in blood

Exploratory objective 3:

To conduct analyses related to furthering the understanding of HIV, immunology, vaccines, and clinical trial conduct.

Exploratory objective 4:
To further evaluate immunogenicity of each vaccine regimen, additional immunogenicity assays may be performed, including on samples from other timepoints, based on the HVTN Laboratory Center assay portfolio.

Exploratory objective 5:

To assess the magnitude of serum antibody neutralization of AdC6 and AdC7 vector both before and after vaccination.

4 COHORT DEFINITION

All data from enrolled participants will be analyzed according to the initial randomization assignment regardless of how many vaccinations they received. In the rare instance that a

participant receives the wrong treatment at a specific vaccination time, the Statistical Analysis Plan (SAP) will address how to analyze the participant's safety and immunogenicity data. Analyses are modified intent-to-treat in those individuals who are randomized but not enrolled do not contribute data and hence are excluded. Because of blinding and the brief length of time between randomization and enrollment—typically no more than 4 working days—very few such individuals are expected.

5 POTENTIAL CONFOUNDERS

Characterization of the safety of the vaccine is susceptible to confounding by adverse events not related to the vaccine that by chance occur more often in one arm of the trial than another. Therefore, analyses involving adverse events will incorporate the reported relationship to product as assessed by HVTN staff.

6 RANDOMIZATION

A participant's randomization assignment will be computer generated and provided to the HVTN CRS pharmacist through a Web-based randomization system. At each institution, the pharmacist with primary responsibility for dispensing study products is charged with maintaining security of the treatment assignments (except in emergency situations as specified in the HVTN MOP).

7 BLINDING

Participants and site staff (except for site pharmacists and other staff designated for pharmacy quality control) will be blinded as to participant treatment group assignments (eg, vaccine or control) but not to study part. Safety assessments will be made in a blinded fashion until the planned unblinding occurs (see protocol **Error! Reference s ource not found.**, **Error! Reference source not found.**, **Error! Reference source not found.**, and **Error! Reference source not found.**).

Study product assignments are accessible to those HVTN CRS pharmacists, DAIDS protocol pharmacists and contract monitors, and SDMC staff who are required to know this information in order to ensure proper trial conduct. Any discussion of study product assignment between pharmacy staff and any other HVTN CRS staff is prohibited excepted for designated staff used solely for the purposes of quality control. The HVTN SMB members also are unblinded to treatment assignment in order to conduct review of trial safety.

When a participant leaves the trial prior to study completion, the participant will be told he or she must wait until all participants are unblinded to learn his or her treatment assignment.

In some cases, the CRS, PSRT, or study sponsor may believe unblinding of the site PI and participant would be appropriate to facilitate the clinical management of an AE or SAE. The HVTN Unblinding MOP specifies procedures for emergency unblinding, and for early unblinding for medical reasons.

8 STATISTICAL ANALYSIS

This section describes the final study analysis, unblinded as to treatment arm assignment. All data from enrolled participants will be analyzed regardless of how many vaccinations they received. All analyses will be performed using SAS and/or R.

8.1 Immunogenicity analysis

8.1.1 General approach

For the statistical analysis of immunogenicity endpoints, data from enrolled participants will be used according to the initial randomization assignment regardless of how many injections they received. Additional analyses may be performed, limited to participants who received all scheduled injections per protocol. Assay results that are unreliable are excluded; results from specimens collected outside of the visit window, from HIV-infected participants post-infection or from participants with study product administration errors due to wrong dose administered, may be excluded. Since the exact date of HIV infection is unknown, any assay data from blood draws 4 weeks prior to an infected participant's last seronegative sample and thereafter may be excluded. If an HIV-infected participant does not have a seronegative sample post-enrollment, then all data from that participant may be excluded from the analysis.

Discrete categorical assay endpoints (eg, response rates) will be analyzed by tabulating the frequency of positive response for each assay by antigen and treatment arm at each timepoint for which an assessment is performed. Crude response rates and difference between treatment arms will be presented with their corresponding 95% confidence interval estimates calculated using the score test method [1].

For quantitative assay data, graphical and tabular summaries of the distributions by antigen, treatment arm, and timepoint will be made. For all primary and secondary immunogenicity endpoints, box plots and plots of estimated reverse cumulative distribution curves will be used for graphical display of all study arms. Typically, the results will be shown for each vaccine arm and for the set of control arms pooled into one group.

Mean or median (if normality assumption severely violated) assay readouts will be compared between arms and bootstrap confidence intervals will be estimated.

More sophisticated analyses employing repeated measures methodology (for example, linear mixed models or marginal mean models fit by generalized estimating equations) may be utilized to incorporate immune responses over several timepoints and to test for differences over time. However, inference from such analyses would be limited by the small sample size of this study. All statistical tests will be 2-sided and will be considered statistically significant if $p \leq 0.05$.

8.1.2 Multivariate display of immunogenicity endpoints

Data visualization techniques may be used to explore the relationship among immunogenicity readouts. The set of readouts may be based on one of the primary endpoints (eg, ICS), on the set of primary endpoints, or on immunogenicity endpoints that also include secondary or exploratory endpoints. To understand the relationship between pairs of readouts, scatter plots may be used when the number of readouts is small or for a larger number of readouts, a heatmap showing the degree of correlation between any two pairs. Principal component analysis (PCA) and associated 'biplots' of the scores and loadings are particularly useful to understand associations between readouts, especially when readouts are correlated [3]. PCA is a method to reduce the dimensionality of the number of readouts to a smaller set of values (principal components) that are normalized linear combinations of the readouts in such a way that the first principal component accounts for the most variability in the data and subsequent components, while maximizing variability, are uncorrelated with each other. A 'biplot' displays the first and second principal component scores and principal component loadings. The x-axis is the value from the first principal component and the y-axis is the second principal component, where each axis label includes the percentage of variation in the total set of readouts captured by the principal component. The top axis is the first principal component loadings and the right axis is the second principal component loadings. An arrow is drawn for each immunogenicity readout (eg, Env-specific CD4+ T-cell polyfunctionality score, Env-specific CD8+ T-cell total magnitude) from the origin to the point defined by its first two principal component loadings. The length of the arrow represents the amount of total variation of the set of readouts captured by the given readout. The direction of an arrow conveys the extent to which the variation of a readout is in the direction of the first or second principal component. The angle between two arrows conveys information about the correlation of the two readouts, with a zero-degree angle denoting perfect correlation and a 90-degree angle denoting no correlation. Each arrow on the biplot is labeled by the immunogenicity readout it represents. A biplot is annotated with key meta-information such as the treatment arm (most common application) or a demographic category. Depending on the application, K-means clustering and hierarchical clustering may also be applied for multivariate graphical display of immunogenicity readouts.

8.1.3 Primary analyses of neutralization magnitude-breadth curves

The area-under-the-magnitude-breadth curve (AUC-MB) to a global panel of viral isolates [4] will be computed for each participant with evaluable neutralization data, as described in [5]. AUC-MB to a vaccine-matched panel may also be computed when the panel consists of at least 3 isolates. Tier 1A viral isolates will not be included in AUC-MB analyses. Dunnett's procedure will be applied with 2-sided alpha = 0.05 to determine which of the [n] vaccine groups have a significantly higher mean AUC-MB than that of the pooled placebo groups, as described in [2] (see their formula (1.1)). This procedure will be applied to construct 95% Cis about the [n] differences in mean AUC-MB for each vaccine regimen versus the pooled placebo

groups (vaccine – placebo), which simultaneously have at least 95% coverage probability.

8.1.4 Analysis of CD4+ and CD8+ T-cell responses as measured by the ICS assay

The analysis of CD4+ and CD8+ T-cell response rates as measured by the ICS assay will be evaluated and compared as described under the general approach. For each T-cell subset, the positivity call for each peptide pool will include a multiple comparison adjustment for the number of peptide pools used in the assay. In general, the Mixture Models for Single-cell Assays (MIMOSA) statistical framework [6] and/or the Fisher's exact test-based positivity criteria will be used. Details of the positivity criteria will be discussed in Section 9. The magnitude of marginal response will be analyzed as described for quantitative data in the general approach section. For each T-cell subset, graphs will be used to display the background-subtracted magnitudes for each participant by protein, treatment arm and timepoint. When 3 or more cytokines are being measured by the ICS assay, the polyfunctionality of ICS responses may also be analyzed as an exploratory endpoint. Besides descriptive plots of the magnitude of polyfunctional responses, the COMPASS (Combinatorial Polyfunctionality analysis of Antigen-Specific T-cell Subsets) statistical framework [7] may also be used to perform joint modelling of multiple T-cell subsets of different cytokine combinations. For example, the functionality score (FS) and the polyfunctionality score (PFS) may be used to summarize the multi-parameter ICS responses.

8.1.5 Analysis of multiplexed immunoassay data

When a small panel of analytes (eg, ≤5) is being assessed in a multiplexed immunoassay, the analysis of response rates and response magnitudes will be evaluated and compared as described under the general approach. Details for calculating a positive response and response magnitude will be provided in Section 9. When a larger panel is being assessed, two approaches may be considered to evaluate the magnitude and breadth of these responses. First, Magnitude–Breadth (M-B) curves maybe employed to display individual- and group-level response breadth as a function of magnitude. Response breadth is defined as the number of antigens, peptides, and/or isolates with associated magnitudes above the assay-specific positivity threshold. Two choices are to compare the M-B curves among vaccine arms, as follows: a non-parametric Wilcoxon rank sum test on the subject-specific AUC-MB or a Kolmogorov-Smirnov type test on the 2 group-average M-B curves. Simulations can be used to obtain 2-sided p-values for the latter test. Second, a weighted-average score-like variable may be constructed to account for the correlations between analytes as an integrate magnitude of responses to multiple analytes. Similar group comparison methods described in the first approach may be adopted. Details of either approach will be described in Section 9.

9 IMMUNOGENICITY TABLES AND FIGURES, BY ASSAY

9.1 Adenovirus Neutralizing Antibody

Similar to other neutralizing antibody assays, the Adenovirus Nab assay relies on incubation of participant samples with the virus(es) of interest, followed by incubation of the virus-sample mixture with infectable host cells expressing a reported gene.

Viral particles of GFP-expressing AdC6 or AdC7 are mixed with twofold serial dilutions (ranging from 1:10 to 1:10,240) of heat-inactivated sera and incubated. After incubation, HEK 293 cells are added per well. Following a 24-hour incubation, cells are fixed with paraformaldehyde and nuclei staining is performed with Hoechst 5 μg/mL. Plates are analyzed under an epifluorescence microscope (BX41, Olympus) in 40× objective lens and approximate percentages of eGFP+ cells are noted for each well. Reported Nab titers correspond to the reciprocal dilutions In which the ratio of GFP-expressing/Hoechst-stained cells reached approximately 50% of the ratio observed in negative controls.

A calibration standard, consisting of mouse serum, is included on every plate and diluted in the same series as participant samples. This standard curve is assessed on each plate for Part A and per day for Part B by the lab and used to adjust titers of samples as needed. Positive controls consist of sera from BALB/c mice primed and boosted with viral particles of GFP-expressing vectors. Negative (virus mixture added with DMEM rather than sera) controls are also included on each plate and assessed internally for precision and consistency with previous validation results. Results are reported by the lab as percent in EGFP+ cells. Study samples as well as controls and standards are run in duplicate. SCHARP will calculate the ID50 titer, using all reported dilutions (10, 20, 40, 80, 160, 320, 640, 1280) and fitting a two-parameter log-logistic (LL.2) model for each participant, visit and isolate (pooling replicates). If all values in titration curves are less than 50% EGFP, ID50 will be censored at >1280 and will be set as 1280 in descriptive analysis. Non-responder will be defined as ID50 titer less than 10, and positive response be ID50 greater or equal to 10. For negative responders, half lowest dilution (= 10/2 = 5) will be assigned as the titer (ID50) and will be used in summary statistics.

For Part A of the study, neutralization titers will be measured on samples from week 0 (visit 2 – baseline) and week 4 (visit 4 – 4 weeks post vaccination). For Part B of the study, neutralization titers will be measured on samples from week 0 (visit 2 – baseline), week 4 (visit 4 – 4 weeks post 1st vaccination), week 16 (visit 7 – 4 weeks post 2nd vaccination).

9.1.1 List of Tables

- Response rate table by lab, virus, visit number (visit month), and treatment arm (as applicable)
- Summary statistics for Ad Nab titers (min, 25th percentile, median, 75th percentile, max, mean, standard deviation) among all participants by lab, virus, visit number (visit month), and treatment arm (as applicable)
- Summary statistics for Ad Nab titers (min, 25th percentile, median, 75th percentile, max, mean, standard deviation) among participants with positive response by lab, virus, visit number (visit month), and treatment arm (as applicable)

9.1.2 List of Graphs

- Boxplots of Ad Nab antibody titers by virus, visit number (visit month), and treatment arm.

- Spaghetti plots of Ad Nab titers by virus, and treatment arm over time.
- (If applicable) Boxplots of Ad Nab titers by virus, visit month (Month 0 and Month 1), and treatment arm with Part A and Part B side-by-side.

9.2 Intracellular Cytokine Staining

Flow cytometry will be used to examine vaccine related HIV-1-specific CD4+ and CD8 responses using a validated ICS assay. A 17-color panel (experiment Assay ID 109; analysis plan 042) was used [8, 9]. The immunogenicity of this regimen will be assessed by intracellular cytokine staining (ICS) at CHIL for all available samples from participants from:

- Part A (n=11), visit 4 (1 month (M) post vaccination)
- Part B (n=20): visits 4 (M1, post first vaccination), 7 (M4, 1 month post the second vaccination) and 9 (M6.5, two weeks post the final vaccination)

Based on results from the above timepoints, others may be assessed, these include:

- Part A (n=11), visit 5 (M6)
- Part B (n=20): visit 10 (M12)

Several criteria will be used to determine if data from an assay are acceptable and cab be statistically analyzed. The blood draw date must have been within the allowable visit window as determined by the protocol and assay results deemed unreliable for analysis by the lab will be excluded from the analysis. Post-infection samples from HIV-infected participants will be excluded. Participants with study administration error due to wrong dose administered will also be excluded. After sample thawing and overnight incubation, the viability of the PBMC must have been 66% or greater for testing to have proceeded. If it was not, a new specimen for that participant at that timepoint will be thawed for testing. If the PBMC viability of the second thawed aliquot is below this threshold, the ICS assay will be not performed, and no data will be reported to the statistical center for the participant and timepoint. For the negative control acceptance criteria, if the average cytokine response for the negative control wells is above 0.1% for either the CD4+ or CD8+ T cells, the sample will be retested. If the retested results are above 0.1%, the data will be excluded from analysis; otherwise, the retest data will be used.

The total numbers of CD4+ and CD8+ T cells must also have exceeded certain thresholds. If the number of CD8+ T cells is < 5,000 or CD4+ T cells is < 10,000 for any of the HIV-1 peptide pools or for one of the negative control replicates for a particular sample, data for that stimulation will be filtered. If both negative control replicates fail for number of T cells, the sample will be retested. If one negative control replicate fail for number of T cells, the negative control replicates with sufficient cells will be used. If both negative control replicates were included, then the sum of total cells and the sum of positive cells were used.

Peptide pools (Any Env) of CD4+ and CD8+ T cells will be calculated as the sum of ENV-1-PTEG-SEQ, ENV-2-PTEG-SEQ.

To assess positivity for a peptide pool within a T-cell subset, a two-by-two contingency table will be constructed comparing the HIV-1 peptide stimulated and negative control data. The four entries in each table are the number of cells positive for IFN-γ and/or IL-2 and the number of

cells negative for IFN-γ and/or IL-2 for both the stimulated and the negative control data. If both negative control replicates are included, then the average number of total cells and the average number of positive cells are used. A one-sided Fisher's exact test is applied to the table, testing whether the number of cytokine-producing cells for the stimulated data is equal to that for the negative control data. Since multiple individual tests (for each peptide pool) are conducted simultaneously, a multiplicity adjustment will be made to the individual peptide pool p-values using the Bonferroni-Holm adjustment method. If the adjusted p-value for a peptide pool is ≤ 0.00001, the response to the peptide pool for the T-cell subset is considered positive. Because the sample sizes (i.e., total cell counts for the T-cell subset) are large, e.g., as high as 100,000 cells, the Fisher's exact test has high power to reject the null hypothesis for very small differences. Therefore, the adjusted p-value significance threshold is chosen stringently (≤ 0.00001). If at least one peptide pool for a specific HIV-1 protein is positive, then the overall response to the protein is considered positive. If any peptide pool is positive for a T-cell subset, then the overall response for that T-cell subset is considered positive.

IFN-γ and/or IL-2-expressing naïve and memory cells (Naïve (N), Central Memory (CM), Effector Memory (EM), and Terminally Differentiated (TEMRA)) will be analyzed in the final report as a percent of CD4 and CD8 T-cells, as well as a percent of IFN-γ and/or IL-2-expressing T-cells. All IFN-γ and/or IL-2-expressing naïve and memory T-cells belong to one of these four types.

9.2.1 Lists of Tables

- Response rate table by lab, T-cell subset, cytokine, peptide pool, visit, visit month, and treatment arm for cells expressing a given cytokine using Fisher's exact test criteria.
- Summary statistics of background-adjusted marginal cytokine response magnitude (i.e., min, mean, median, max) among all participants (positive and negative responders) for T-cell subset, cytokine, peptide pool, visit, and treatment arm.
- Summary statistics of background-adjusted marginal cytokine response magnitude (i.e., min, mean, median, max) among responders for T-cell subset, cytokine, peptide pool, visit, and treatment arm.
- Response rate and/or response magnitude comparison of treatment arms.
    a. Treatment 1 vs. Treatment 4 at 4 weeks after 1st vaccination
    b. Treatment 2 vs. Treatment 5 at 4 weeks after 1st vaccination
    c. Treatment 4 vs. Treatment 5 at 4 weeks after 1st vaccination, 4 weeks after 2nd vaccination, 2 weeks after 3rd vaccination, and 6 months after 3rd vaccination

9.2.2 Lists of Graphs

- Boxplots of background-adjusted marginal cytokine response magnitude by T-cell subset, HIV protein, visit month, and treatment arm using Fisher exact test.

- Spaghetti plots of background-subtracted percent of cells expressing a given cytokine over time (as applicable), by T cell subset, antigen, cytokine, and treatment arm
- (If applicable) Boxplots of background-adjusted marginal cytokine response magnitude by T-cell subset, HIV protein, visit month (Month 0 and Month 1), and treatment arm using Fisher exact test. with Part A and Part B side-by-side
- Line plots of background-subtracted percent of T cells expressing naïve and memory cell subsets, by T cell subset, memory cell subset, visit month, and treatment group (4 rows of memory cell subsets per page).
- Line plots of relative percent of naïve and memory cell subsets out of positive T cells expressing IFN-γ and/or IL-2, by T cell subset, memory cell subset, visit month, and treatment group (4 rows of memory cell subsets per page).

9.3 Neutralizing Antibody

Assays will be performed on cryopreserved serum samples obtained 4 weeks after the 1st vaccination (M1) for Part A. For Part B, serum samples will be obtained 4 weeks after the 1st vaccination (M1), 4 weeks after the 2nd accination (M4) and 2 weeks after the 3rd vaccination (M6.5). Assays will also be performed on baseline samples (M0) for Part A and Part B.

Neutralizing antibodies against tier 1 and tier 2 strains of HIV-1 will be measured in TZM-bl cells as a function of a reduction in Tat-induced luciferase reporter gene expression after a single round of infection with molecularly cloned, Env-pseudotyped viruses [10]. TZM-bl (also called JC57BL-13) is a HeLa cell clone that was engineered to express CD4 and CCR5 [11] and to contain integrated reporter genes for firefly luciferase and E. coli ß-galactosidase under control of an HIV-1 long terminal repeat (LTR) [12]. The cells are highly permissive to infection by most strains of HIV, including primary HIV-1 isolates and molecularly cloned Env-pseudotyped viruses. DEAE-dextran (Diethylaminoethyl-Dextran) is used in the medium during neutralization assays to enhance infectivity. Expression of the reporter genes is induced in trans by viral Tat protein soon after infection. Luciferase activity is quantified by luminescence and is directly proportional to the number of infectious virus particles present in the initial inoculum. The assay is performed in 96-well culture plates for high throughput capacity. Use of a clonal cell population provides enhanced precision and uniformity. The assay has been formally optimized and validated for single-round infection with either uncloned or molecularly cloned Env-pseudotyped viruses produced by transfection in 293T cells [13].

All designated serum samples will be assayed against Tier 1 and Tier 2 strains of virus by starting with a 1:10 dilution of serum to obtain a neutralizing antibody titer. The starting dilution of serum can be adjusted by the primary investigator to ensure obtainment of a neutralizing antibody titer. Titers will be defined as the serum dilution that reduces RLUs by 50% relative to the RLUs in virus control wells after subtraction of background. Each plate will contain 8 wells of cell controls (cells only) and 8 wells of virus controls (cell + virus only). A broadly neutralizing antibody PGDM1400 will serve as a positive control and will be tested on at least one plate in a series. One series of plates constitutes all plates set-up in a single session by a single technician. Mandatory assays with visit 4 and 104 samples (1 month post 1st vaccination) and visit 107 and 109 (1 month post 2nd and 2 weeks post 3rd vaccination respectively) will measure neutralization

titers in TZM-bl cells against the Env-pseudotyped vaccine strains (Du422.1, Du172.17 and CH0505TF) and a virus that exhibits a tier 1A neutralization phenotype (MW965.26). Additional neutralization assays may also be performed with samples against a clade C panel of tier 2 Env-pseudotyped viruses (ZM233M, Ce703010010, 2759058, ZM215F, So431, Ce704810053, 3728, 2969249, B005582, CAP382, Ce2103, and Ko243) and/ or against viruses from a global panel of heterologous tier 2 Env-pseudotyped viruses (246F3, Ce1176, CNE55, X1632, Ce0217, BJOX2000, 25710, TRO11, CH119, X2278, CNE8 and 398F1) [14] Additional assays may be performed in TZM-bl cells to measure neutralization titers against other tier 1 and tier 2 viruses not listed above.

A response is considered positive if the neutralization titer is 10 or above, the starting dilution, which is the limit of detection of the assay. Magnitude of response will be measured by the natural log of the ID50 and ID80 titer.

9.3.1 List of Tables

- Response rate table by lab, inhibitory dilution, isolate, part, visit number (visit month), and treatment arm.
- Summary statistics (i.e., min, mean, median, max) among all participants (positive and negative responders) by lab, inhibitory dilution, isolate, part, visit number (visit month), and treatment arm, upon request.
- Summary statistics (i.e., min, mean, median, max) among responders by lab, inhibitory dilution, isolate, part, visit number (visit month), and treatment arm, upon request.
- Response rate and/or response magnitude comparison of treatment arms.
    a. Treatment 1 vs. Placebo 3
    b. Treatment 2 vs. Placebo 3
    c. Treatment 1 vs. Treatment 2
    d. Treatment 4 vs. Placebo 6
    e. Treatment 5 vs. Placebo 6
    f. Treatment 4 vs. Treatment 5
    g. Treatment 1 vs. Treatment 4 at 4 weeks after 1st vaccination
    h. Treatment 2 vs. Treatment 5 at 4 weeks after 1st vaccination

9.3.2 List of Graphs

- Boxplots of neutralizing antibody titers by part, isolate, inhibitory dilution (ID50/ID80), visit number (visit month), and treatment arm.
- (If applicable) Boxplots of neutralizing antibody titers by isolate, inhibitory dilution (ID50/ID80), visit month (Month 0 and Month 1), and treatment arm with Part A and Part B side-by-side

9.4 Binding Antibody Multiplex Assay

Serum HIV-1-specific IgG responses 1:50 against vaccine matched antigens (Du422 gp140, Du172 gp140) will be performed on Part A at visit 2 (Month 0; Baseline), visit 4 (Month 1; 4 weeks post 1st vaccination), Part B at visit 2 (Month 0; Baseline), visit 4 (Month 1; 4 weeks post 1st vaccination), visit 7 (Month 4; 4 weeks post 2nd vaccination), and visit 9 (Month 6.5; 2 weeks post 3rd vaccination). Additional reagents and material are listed below.

Panel	Antigen
	CH505TF D7 gp120
	CH505TF D7 gp120d371
	CH505TFD8N 156KN160K_avi/2931i Mon
	Con S gp140
	Con 6 gp120
	gp41
	gp70_CH505TF_V1V2_avi/293F
	gp70_CH505TF_V1V2_N156KN160K_avi/293F
Clade C gp120 panel	1394C9_G1.D11gp120.avi
	1428_D11gp120.avi/293F
	1641A7_D11gp120.avi/293F
	CAP210_D11gp120.avi/293F
	CAP45_D11gp120.avi/293F
	Ce0042_D11gp120.avi/293F
	CH505TF_D7gp120.avi/293F
	Du156_D11gp120.avi/293F
	96ZM65.1D11gp120.avi
	TV1c8_D11gp120.avi/293F
gp70 V1V2 Clade C panel	gp70-BF1266_431a_V1V2
	gp70-7060101641 V1V2
	gp70-96ZM651.02 V1V2
	gp70-001428.2.42 V1V2
	gp70-TV1.21 V1V2
	gp70-Ce1086_B2 V1V2
	gp70-CAP45.2.00.G3 V1V2
	gp70-1394C9G1 V1V2
	gp70-1012.11.TC21.3257 V1V2
	gp70-1051.12.C22 V1V2
	gp70-Ce1176 V1V2
	gp70-Ce70401042_2ES V1V2
	gp70-ConC V1V2
	gp70-Du156.12.V1V2

	1086C_D7gp120.avi/293F

The assay readout is from a Bio-Plex instrument (Bio-Rad). The Bioplex software provides 2 readouts: a background-subtracted mean fluorescent intensity (MFI), where background refers to a plate level control (i.e., a blank well run on each plate), and a concentration based on a standard curve. Net MFI is MFI minus Neg, where 'Neg' refers to a sample-specific background measure. Samples from post-enrollment visits are declared to have positive responses if they meet three conditions: (1) the Net MFI values are ≥ antigen-specific cutoff at the 1:50 dilution level for IgG (based on the 95th percentile of the baseline visit serum samples and at least 100 MFI minus Blank), (2) the Net MFI values are greater than 3 times the baseline (day 0) Net MFI values, and (3) the MFI values are greater than 3 times the baseline MFI values.

Differential Binding Response

Differential binding response will be evaluated among a particular set of wild type and mutant type antigens as shown below.

Wild Type	Matched Mutant
CH0505_CON D7gp120_avi/293F/Mon	CH505TF D7gp120d371_avi/293F/Mon

Differential response criteria are the following:

1. Wild type MFI* (net MFI=MFI – Blank) values are ≥ antigen-specific cutoff at the screening dilution level (based on the 95th percentile of the baseline visit samples and at least 100 MFI*)
2. Wild type MFI* values are greater than 3 times the baseline (day 0) MFI* values
3. Wild type MFI values are greater than 3 times the baseline MFI values.

The first 3 criteria stated above can be concluded as "WT delta response call" is positive at screening dilution. Then, among the titration series' dilutions, select the dilution where most of both Wild Type and Mutant Type analytes' Net MFI fall in the assay linear range [100, 22000] net MFI at a time point. This dilution selection will be antigen pair and timepoint dependent, in other words, the dilution selection can be varied by antigen pair or timepoints. if there are more than one dilution having the same number of data points in linear range, then select the lower dilution factor. At the selected dilution:

4. MFI ratio (wild type/mutant type) ≥ 2.5, and wild type at least 250 MFI
5. MFI* ratio (wild type/mutant type) ≥ 2.5, and wild type at least 250 MFI*

When calculating the MFI and MFI* ratios, no ceiling is applied to MFI or MFI*, e.g. MFI* > 22k are not truncated. MFI and MFI* values <=1 are set to 1.

If sufficient immunogenicity is observed, samples may be titrated to calculate antibody titrations. Titrations are quantified by EC50/AUC. The geometric mean will be compared between the placebo and vaccine groups. The overall difference in geometric mean titer between groups at each time point assays will be compared.

9.4.1 List of Tables

- Response rate table by lab, isotype, antigen, visit number (visit month), and treatment arm
- Summary statistics of net MFI values (i.e., min, mean, median, max) among all participants (positive and negative responders) by lab, isotype, antigen, visit number (visit month), and treatment arm, upon request.
- Summary statistics of net MFI values (i.e., min, mean, median, max) among responders by lab, isotype, antigen, visit number (visit month), and treatment arm.
- Summary statistics of binding antibody titers (AUC) (i.e., min, mean, median, max) among all participants (positive and negative responders) by lab, isotype, antigen, visit number (visit month), and treatment arm, upon request.
- Summary statistics of binding antibody titers (AUC) (i.e., min, mean, median, max) among responders by lab, isotype, antigen, visit number (visit month), and treatment arm.
- Comparison of response rates between different doses (T1 vs T4, T2 vs T5) at 4 weeks post one vaccination, by product, isotype, and antigen
- Comparison of net MFI values between different doses (T1 vs T4, T2 vs T5) at 4 weeks post one vaccination, by isotype, and antigen among all participants and among positive responders.
- Comparison of binding antibody titers (AUC) between different doses (T1 vs T4, T2 vs T5) at 4 weeks post one vaccination, by isotype, antigen, and visit among all participants and among positive responders.
- Comparison of response rates between different sequential administration in Part B (T4 vs T5) at 4 weeks post the 2nd vaccination and 2 weeks post the 3rd vaccination, by product, isotype, and antigen
- Comparison of net MFI values between different sequential administration in Part B (T4 vs T5) at 4 weeks post the 2nd vaccination and 2 weeks post the 3rd vaccination, by isotype, antigen, and visit among all participants and among positive responders.
- Comparison of binding antibody titers (AUC) between different sequential administration in Part B (T4 vs T5) at 4 weeks post the 2nd vaccination and 2 weeks post the 3rd vaccination, by isotype, antigen, and visit among all participants and among positive responders.
- Differential binding response rates by isotype, differential binding antigen pair, visit, and treatment arm.
- Summary statistics of differential binding response ratio (i.e., min, mean, median, max) by lab, isotype, antigen pair, visit number (visit month), and treatment arm.
- Summary statistics of epitope binding percentage (i.e., min, mean, median, max) by lab, isotype, antigen pair, visit number (visit month), and treatment arm.

9.4.2 List of Graphs

- Bar and Boxplots of net MFI values by isotype, antigen, visit number (visit month), and treatment arm.
- Spaghetti plots of net MFI values over time in Part B, by isotype, antigen, and treatment arm
- Bar and Boxplots of binding antibody titers (AUC) by isotype, antigen, visit number (visit month), and treatment arm.

- Spaghetti plots of binding antibody titers (AUC) over time in Part B, by isotype, antigen, and treatment arm
- Bar and boxplots of differential binding response rates and the distribution of differential binding response rates by isotype, analyte, visit (visit month) and treatment arm.
- Scatterplots of differential binding pairs net response by visit month.

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