STATISTICAL ANALYSIS PLAN FOR HVTN IMMUNOGENICITY

Protocol HVTN *303* (v2.0)

A Phase 1, Open-Label Clinical Trial to Evaluate Safety, Tolerability, and Immunogenicity of Adjuvanted HIV-1 Fusion Peptide Conjugate Vaccine (VRC-HIVVCP0108-00-VP) Alone or in Prime-Boost Regimens with Adjuvanted HIV-1 Envelope Trimer 4571 (VRC-HIVRGP096-00-VP) and HIV-1 Trimer 6931 (VRC-HIVRGP0106-00-VP) Vaccines in Healthy Adults

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Statistical Analysis Plan for Immunogenicity

Protocol: HVTN 303 (v2.0)

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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	Add circulating immune complex panel assay
3.0	Add MSD cytokine assay
4.0	Modify MSD cytokine assay
5.0	Add NAb assay
6.0	Add BCP assay
7.0	Add EMPEM assay
8.0	Modify EMPEM assay
9.0	Modify MSD binding assay

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1 OVERVIEW

The following describes the Statistical Analysis Plan (SAP) for the analysis of immunogenicity data from HVTN 303 for Protocol Team (PT) reports for immunogenicity data, and the FSR for Immunogenicity.

2 PROTOCOL SUMMARY

2.1 Title

A Phase 1, Open-Label Clinical Trial to Evaluate Safety, Tolerability, and Immunogenicity of Adjuvanted HIV-1 Fusion Peptide Vaccine (VRC- HIVVCP0108-00-VP) Alone or in Prime-Boost Regimens with Adjuvanted HIV-1 Envelope Trimer 4571 (VRC-HIVRGP096-00-VP) and HIV-1 Trimer 6931 (VRC-HIVRGP0106-00-VP) Vaccines in Healthy Adults.

2.2 Design

This is a phase 1, open-label, dose-escalation study to evaluate the dose, safety, tolerability and immunogenicity of adjuvanted HIV-1 Fusion Peptide (FP) conjugate vaccine (FP8v1-rTTHC) alone or in prime-boost regimens with adjuvanted HIV-1 Trimer 4571 and adjuvanted HIV-1 Trimer 6931. The primary hypothesis is that FP8v1-rTTHC, HIV-1 Trimer 4571, and HIV-1 Trimer 6931 adjuvanted vaccines are safe and tolerable when administered alone and when co-administered with HIV-1 Trimer 4571, in prime-boost regimens.

2.3 Study products

- FP8v1-rTTHC (FP conjugate vaccine) is an HIV-1 fusion peptide conjugated to recombinant tetanus toxoid heavy chain fragment C (rTTHC) via sulfo-SIAB chemical linker. FP8v1 corresponds to the amino-terminal eight residues of the most prevalent HIV fusion peptide sequence. FP conjugate vaccine will be provided at a 1-milligram (mg)/milliliter (mL) concentration in 3-mL glass vials filled to 0.7 mL.
- HIV-1 Trimer 4571 (Trimer 4571) is a synthetic soluble HIV-1 envelope product that consists of an HIV-1 envelope (Env) trimer variant, derived from clade A, strain BG505. Trimer 4571 is provided at a 500-mcg/mL concentration in 3-mL glass vials filled to 1.2 mL
- HIV-1 Trimer 6931 (Trimer 6931) is a synthetic soluble HIV-1 envelope product that consists of an HIV-1 envelope (Env) trimer variant, derived from consensus clade C sequence (ConC). Trimer 6931 will be provided at a 1-mg/mL concentration in 3-mL glass vials filled to 1.2 mL
- Adjuplex is the adjuvant and will be provided in a sterile, pyrogen-free, homogeneous suspension at 0.7 mL in 3-mL glass vials. Adjuplex will be mixed with study products in the pharmacy during preparation prior to vaccination at a 20% dose by volume
- PBS (labeled as Phosphate Buffered Saline pH 7.2): Diluent

2.4 Study population

Healthy adults aged 18 to 50 years, inclusive

2.5 Study plans and schema table

This study has two parts. Part A will evaluate the safety, tolerability, and immunogenicity of single doses of the FP conjugate, Trimer 4571 and Trimer 6931 vaccines, in a dose-escalation design. Each product must be assessed as safe prior to use in Part B. Trimer 4571 with alum adjuvant has been previously evaluated in humans but will be tested in Part A with Adjuplex. Part B will evaluate the safety, tolerability, and immunogenicity of FP conjugate prime, Trimer 4571 prime, or an FP plus

Trimer 4571 prime, all followed by subsequent doses of Trimer 4571, Trimer 6931 and both Trimers combined. Study vaccines will be administered intramuscularly (IM) via needle and syringe in two injection sites. The study schema is below:

Table 2-1 Schema

Part A: Dose Escalation**											
Group	**N**	**W0**									
1	3	25 mcg FP conjugate vaccine									
2	3	200 mcg FP conjugate vaccine									
3	3	100 mcg Trimer 6931									
4	3	200 mcg Trimer 6931									
5	3	200 mcg Trimer 4571									
Part A Total	**15***										
Part B: Prime Boost Regimen**											
Group	**N**	**W0**	**W4**	**W8*** **	**W12*** **	**W20*** **	**W24*** **	**W32*** **	**W36*** **	**W44*** **	**W48*** **
6	~~15~~ 10	200 mcg Trimer 4571			~~200 mcg Trimer 4571~~		~~200 mcg Trimer 6931~~		~~100 mcg Trimer 4571 + 100 mcg Trimer 6931~~		~~100 mcg Trimer 4571 + 100 mcg Trimer 6931~~
7	~~15~~ 10	200 mcg FP conjugate vaccine	200 mcg FP conjugate vaccine	~~200 mcg FP conjugate vaccine~~	~~200 mcg Trimer 4571~~		~~200 mcg Trimer 4571~~		~~200 mcg Trimer 6931~~		~~100 mcg Trimer 4571 + 100 mcg Trimer 6931~~
8	~~15~~ 9	200 mcg FP conjugate vaccine + 200 mcg Trimer 4571	200 mcg FP conjugate vaccine + 200 mcg Trimer 4571	~~200 mcg FP conjugate vaccine + 200 mcg Trimer 4571~~		~~200 mcg Trimer 6931~~		~~100 mcg Trimer 4571 + 100 mcg Trimer 6931~~		~~100 mcg Trimer 4571 + 100 mcg Trimer 6931~~	
Part B total	**~~45~~§ 29**										
Overall Total	**~~60~~† 44**										

Table 1-1 Footnotes:

**Adjuplex adjuvant will be mixed with all study products in Part A and Part B at 20% by volume in the pharmacy during product preparation for all vaccinations. Once mixed, all study injections will be divided into 2 syringes, and each syringe will be administered intramuscularly to one of the deltoids.

* In Part A, up to 20 participants may be enrolled if needed for safety or immunogenicity evaluations. Additional participants may be enrolled to ensure the availability of 2-week safety data from at least 3 participants per group.

§ In Part B, up to 50 participants may be enrolled if needed for safety or immunogenicity evaluations. Additional participants may be enrolled to ensure the availability of 2-week safety data from at least 15 participants per group.

† Total up to 70 participants can be enrolled if needed for safety or immunogenicity evaluations.

Notes:

***Vaccination in Part B starting from Week 8 onwards did not take place.

Actual Ns for Group 6, 7 and 8 were 10, 10 and 9 respectively. In Part B total 29 participants were enrolled

Part A of the study may begin with direct enrollment of participants into the following groups simultaneously:

• Group 1 with no more than 1 participant enrolled per day for the 3 participants.

• Group 3 with no more than 1 participant enrolled per day for the 3 participants.

After Groups 1 and 3 have been fully enrolled, the study will be placed on a safety hold. No additional enrollments will proceed until the Protocol Safety Review Team (PSRT) has determined it is safe to do so. Once all of the reactogenicity and 2-week safety data from at least 6 participants have been submitted to the database, the PSRT must assess the accumulated product-specific data as showing no significant safety concerns before proceeding with enrollment of Groups 2, 4 and 5. If the PSRT has determined it is safe to proceed after reviewing data from Groups 1 and 3, the following groups may begin simultaneously:

• Group 2 with no more than 1 participant enrolled per day for the 3 participants

• Group 4 with no more than 1 participant enrolled per day for the 3 participants

• Group 5 with no more than 1 participant enrolled per day for the 3 participants

Once Groups 2, 4 and 5 have been fully enrolled, the study will be placed on a safety hold before proceeding with Part B. Once all of the reactogenicity and 2-week safety data from at least 9 participants have been submitted to the database, the PSRT must assess the accumulated product-specific data as showing no significant safety concerns before proceeding with enrollment of Part B.

Part B enrollments may only proceed if no safety concerns have been identified for any of the product administrations in Part A of the study.

If at any time there is insufficient data to conduct a formal PSRT Safety Review because of participant discontinuations from the study before sufficient data are collected, then additional participants may be enrolled into that group to acquire the requisite data on the required number of participants specified above. Moreover, the PSRT may recommend additional participants be enrolled into a given treatment group if additional safety evaluations are requested.

Consultation with the HVTN Safety Monitoring Board (SMB), Institutional Review Board (IRB) and Food and Drug Administration (FDA), if needed, as specified by study pause criteria (per Section 9.5.1), will occur if indicated.

2.6 Duration per participant

For participants in Part A (Groups 1-5): 52 weeks of scheduled clinic visits

~~For participants in Part B (Groups 6 and 7): 100 weeks of scheduled clinic visits~~

~~For participants in Part B (Group 8): 96 weeks of scheduled clinic visits~~

On January 13, 2023, a protocol memo was distributed informing the clinical research sites that all vaccinations in HVTN 303 were permanently discontinued. Procedures specified for remaining follow-up visits have been revised. Duration for Part A participants remained unchanged. Duration for Part B participants (for Groups 6 -8) is 56 weeks.

2.7 Estimated total study duration

~~Total study duration is 36 months (includes enrollment, planned safety holds and follow-up).~~

Following cessation of vaccination and reduction of follow-up duration the estimated total study duration is reduced to approximately 18 months.

2.8 Study Sites

HVTN Clinical Research Sites (HVTN CRSs) to be specified in the Site Announcement Memo.

3 OBJECTIVES AND ENDPOINTS

3.1 Primary objectives and endpoints

Primary objective 1:

To evaluate the safety and tolerability of the following regimens in healthy adults:

- Adjuvanted FP conjugate vaccine administered IM at a dose of 25 or 200 mcg,
- Adjuvanted Trimer 6931, administered IM at a dose of 100 or 200 mcg,
- Adjuvanted Trimer 4571 administered IM at 200 mcg, or
- Prime-boost vaccination regimens of FP conjugate, Trimer 4571, and Trimer 6931 vaccines.

Primary endpoint 1:

Local and systemic reactogenicity signs and symptoms, laboratory measures of safety, and adverse and serious adverse events.

SAEs, medically attended adverse events (MAAEs), adverse events of special interest (AESIs) and AEs leading to early participant withdrawal or permanent discontinuation which will be collected throughout the study and for 12 months following any receipt of study product. Additionally, all adverse events will be collected for 28 days after any receipt of study vaccination. All safety lab related adverse events will be collected throughout duration of study.

Primary objective 2:

To evaluate the ability of FP-conjugate and Trimer 4571 vaccines to elicit FP-specific binding antibodies in Part B participants.

Primary endpoint 2:

Magnitude and response rate of serum antibody binding of FP and envelope trimer antigens as measured by the MSD assay 2 weeks after the last vaccination.

3.2 Exploratory objectives

Exploratory objective 1:

Mapping of FP-specific serum neutralizing activity

Exploratory objective 2:

To evaluate the ability of the vaccine regimen to elicit early FP broad neutralizing antibody memory B-cell lineages.

Exploratory objective 3:

To evaluate the humoral and cellular immune response to vaccination regimens including FP-conjugate vaccine and Trimer 4571 to compare responses between the regimens.

Exploratory objective 4:

To conduct analyses related to furthering the understanding of HIV, immunology, vaccines, and clinical trial conduct. To further evaluate immunogenicity of each vaccine regimen, additional immunogenicity assays may be performed in a subset of participants, including on samples from other timepoints, based on the HVTN Laboratory Assay Algorithm.

4 COHORT DEFINITION

Recruitment will target 60 (up to 70) healthy adult participants 18 to 50 years of age. The primary goal of this study is to identify safety concerns that may be associated with the study products.

Since enrollment is concurrent with receiving the first study vaccination, all enrolled participants will provide some safety data. For immunogenicity analyses, it is possible that data may be missing for various reasons, such as participants terminating from the study early, problems in shipping specimens, low cell viability of processed PBMCs, or high assay background.

5 RANDOMIZATION

In Part A, Groups 1 and 3 will be randomized and will enroll no more than 1 participant per day for 3 participants per group. Contingent on the safety data from Groups 1 and 3, Groups 2, 4, and 5 will be randomized and may enroll simultaneously with no more than 1 participant per day for 3 participants for each group. Contingent on the safety data from Groups 1-5 in Part A, Groups 6-8 in Part B will be randomized and stratified by whether or not participants will be willing to consent to leukapheresis. A maximum of 7 participants per group that do NOT consent to leukapheresis collection will be enrolled. 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 manual of procedures [MOP]).

6 BLINDING

Participants and site staff will be unblinded to participants' group assignments. Laboratory program staff will be unblinded to whether a sample is from Part A or Part B but will remain blinded to the treatment assignment within Part A or Part B during assay analysis.

7 STATISTICAL ANALYSIS

For the statistical analysis of immunogenicity endpoints, data from enrolled participants in Part B will be used according to the initial randomization assignment regardless of how many injections the participants received. Additional analyses may be performed, limited to participants who received all scheduled injections per protocol. Assay results from specimens collected outside of the visit window, or from HIV-infected participants collected post infection, 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 will be presented with their corresponding 95% CI estimates calculated using the score test method [1]. Barnard's or Fisher's exact tests, as specified in the SAP, will be used to compare the response rates of any 2 vaccine arms, with a significant difference declared if the 2-sided p-value is ≤ 0.05. In general, Barnard's is preferred since, under most circumstances, it is more powerful than Fisher's [2].

In addition to response rate estimates for each timepoint, the probability of observing at least 1 positive response by a given timepoint and the probability of observing more than 1 positive response by a given timepoint will be estimated, with corresponding CIs, for each vaccine arm using maximum likelihood-based methods [3].

For quantitative assay data (eg, the vaccine-induced serum neutralizing antibody [nAb] as measured by the TZM-bl assay, HIV-specific serum IgG binding magnitude, breadth, and avidity to cross-clade panels of gp120 and V1V2 and to V3, CD4i, gp41 and gp41 immunodominant region [IDR] as assessed by the binding antibody multiplex assay [BAMA] or CD4+ T-cell responses to Gag and Env as assessed by ICS), 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 of the study arms. Typically, the results will be shown for each vaccine arm.

The difference between arms at a specific timepoint will be tested with a nonparametric Wilcoxon rank sum test if the data are not normally distributed and with a 2-sample t-test if the data appear to be normally distributed. To test for differences among all vaccine arms, first a Kruskal-Wallis rank test or an F-test (depending on the normality assumption) will be used to test for overall differences. Secondly, if the overall test is significant at the 2-sided 0.05 level, then individual tests comparing between vaccine arms will be done. If rank-based tests are used, then the tests will be inverted to construct Hodges-Lehmann point estimates and 2-sided (1-0.05/# of comparisons) × 100% CIs about the differences in location centers of the number of comparisons between vaccine arms. If rank-based tests are used, then the tests will be inverted to construct Hodges-Lehmann point estimates and 2-sided (1-0.05/# of comparisons) × 100% CIs about the differences in location centers of the number of comparisons between vaccine arms. When all pair-wise comparisons between the multiple vaccine arms are of interest, the Tukey procedure [4] will be used. If only specific comparisons between pairs of the multiple vaccine arms are of interest, the Holm-Bonferroni procedure will be used. An appropriate data transformation (eg, log10 transformation) may be applied to better satisfy assumptions of symmetry and homoscedasticity (constant variance). Significance of the differences between pairs will be evaluated using 2 procedures, first based on whether the simultaneous 95% CIs exclude zero and secondly, based on whether the nominal (unadjusted) 95% CIs exclude zero.

Some immunologic assays have underlying continuous or count-type readout that are dichotomized into responder/nonresponder categories (eg, nAb, BAMA, ICS). If treatment arm differences for these assays are best summarized by a mixture model, then either Lachenbruch's test statistic [5] or an alternative 2-part test [6] (as defined in the SAP) will be used to evaluate the composite null hypothesis of equal response rates in the 2 arms and equal response distributions. Lachenbruch's test statistic equals the square of a binomial Z-statistic for comparing the response rates plus the square of a Wilcoxon statistic for comparing the response

distributions in the subgroup of responders. A permutation procedure is used to obtain a 2-sided p-value. For estimation, differences in response rates between arms will be estimated using the methods described above, and in the subgroup of positive responders, differences in location parameters between arms will be estimated using the methods described above.

More sophisticated analyses employing repeated measures methodology (for example, linear mixed models or marginal mean models fit by generalized estimating equations [GEE]) 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 $p < 0.05$.

Based upon previous HVTN trials, missing 15% of immunogenicity results for a specific assay is common due to study participants terminating from the study early, problems in shipping specimens, or low cell viability of processed PBMCs. To achieve unbiased statistical estimation and inferences with standard methods applied in a complete-case manner (only including participants with observed data in the analysis), missing data need to be missing completely at random (MCAR). Following the most commonly used definition, MCAR assumes that the probability of an observation being missing does not depend on any participant characteristics (observed or unobserved). When missing data are minimal (specifically if no more than 20% of participants are missing any values), then standard complete-case methods will be used, because violations of the MCAR assumption will have little impact on the estimates and hypothesis tests.

If a substantial amount of immunogenicity data are missing for an endpoint (at least 1 value missing from more than 20% of participants), then using the methods that require the MCAR assumption may give misleading results. In this situation, analyses of the immunogenicity endpoints at a specific timepoint will be performed using parametric generalized linear models fit by maximum likelihood. These methods provide unbiased estimation and inferences under the parametric modeling assumptions and the assumption that the missing data are missing at random (MAR). MAR assumes that the probability of an observation being missing may depend upon the observed responses and upon observed covariates, but not upon any unobserved factors. Generalized linear models for response rates will use a binomial error distribution and for quantitative endpoints, a normal error distribution. For assessing repeated immunogenicity measurement, linear mixed effects models will be used. If the immunological outcomes are left- and/or right-censored, then the linear mixed effects models of Hughes [7] will be used because they accommodate the censoring. In addition, secondary analyses of repeated immunogenicity measurements may be done using weighted GEE [8] methods, which are valid under MAR. All of the models described above in this paragraph will include as covariates all available baseline predictors of the missing outcomes.

8 IMMUNOGENICITY TABLES AND FIGURES, BY ASSAY

8.1 MSD Binding Assay

Serum samples collected in HVTN303 will be assayed using the Meso Scale Discovery Immunogenicity Assay (MSD) to assess serum HIV-1-specific IgG responses against fusion protein (FP), Trimer 4571, and Trimer 6931.

The objectives/endpoints addressed are as follows:

Primary objective 2: To evaluate the ability of FP-conjugate and Trimer 4571 vaccines to elicit FP-specific binding antibodies in Part B participants.

Primary endpoint 2: Magnitude and response rate of serum antibody binding of FP and envelope trimer antigens as measured by the MSD assay 2 weeks after the last vaccination.

Standard MSD 384 well streptavidin coated SECTOR®Imager 6000 plates will be blocked with 35 µL of 5% (W/V) MSD Blocker A and incubated for 1 hr. at room temperature (RT) on a Heidolph Titramax 100 vibrational plate shaker at 650 rpm. The plates will be washed thrice with 0.05%Tween PBS (wash buffer) and will be coated with biotinylated antigens at an optimized concentration for 1 hour. 1% MSD Blocker A will be used as the diluent in the assay. Duplicate wells of serially diluted test samples will be prepared in dilution plates. After 1 hour of incubation, the plates will again be washed with the wash buffer and the serial dilution samples plates will be added to MSD plates. After an hour of incubation with the samples, the plates will be washed again, and SULFO-TAG conjugated anti-human secondary detection antibody will be used for detection at an optimized concentration. After an additional hour of incubation, the unbound secondary detection antibody will be washed off the plates and the plates will be read using 1X MSD Read Buffer on the MSD Sector Imager S600.

All samples will be testing in one batch. The readout will be AUC (Area Under the Curve) calculated from the serial dilution of the sample.

Binding MSD will be performed on all serum samples collected at baseline, 2 weeks post the first vaccination from participants in Group 5 in Part A and Groups 6-8 in Part B, and 2 weeks post the 2nd vaccination from participants in Groups 7-8 who received the 2nd vaccination.

Part A (group 5, 1 vaccination):

- V2 [W0, baseline]
- V4 [W2, 2 wk post 1 vacc]

Group 6 (1 vaccination):

- V101 [W0, baseline]
- V103 [W2, 2 wk post 1st vacc]

Group 7 (1 or 2 vaccinations):

- V101 [W0, baseline]
- V103 [W2, 2 wk post 1st vacc]
- V106 [W6, 2 wk post 2nd vacc]

Group 8 (1 or 2 vaccinations):

- V101 [W0, baseline]
- V103 [W2, 2 wk post 1st vacc]
- V106 [W6, 2 wk post 2nd vacc]

Positivity call will be made based on two different methods: 1) use the cutoffs from the lab (Trimer 6931 = 227 AUC; Trimer 4571 = 354 AUC; Fusion Peptide = 867 AUC) based on the 80 naïve samples (the 95th percentile) 2) use the cutoffs (mean + 3*SD of AUC to each analyte) based on the 80 naïve samples after filtering out %CV≥30% plus the baseline values from this study (Trimer 6931 = 602 AUC; Trimer 4571 = 1039 AUC; Fusion Peptide = 1121 AUC). The first positive call criterion is expected to have an

5% false positive. The second positive call criterion is more conservative with none or less false positive than the first criterion.

Group 5 and Group 6 will be combined in the summary of IgG response rate and magnitudes at 2 weeks post the 1st vaccination and comparisons with Groups 7 and 8.

The following comparisons of IgG response rate and magnitudes will be done:

- The response rate will be compared between Group 5+6 vs Group 7 vs Group 8 at 2 weeks post the 1st vaccination and between Group 7 vs Group 8 at 2 weeks post the 2nd vaccination. The comparisons will be done using Barnard's test.
- The response magnitudes will be compared between Group 5+6 vs Group 7 vs Group 8 at 2 weeks post the 1st vaccination and between Group 7 and Group 8 at 2 weeks post the 2nd vaccination. The comparisons will be done among positive responders only as well as among all participants regardless the positive calls using Wilcoxon rank sum test.
- The response rate will be comparison between 2 weeks post the 2nd vaccination vs the 1st vaccination within Group 7 and Group 8. The comparison will be done using McNamar's test.
- The response magnitudes will be compared between 2 weeks post the 2nd vaccination vs the 1st vaccination within Group 7 and Group 8. The comparison will be done among the participants who received both vaccinations regardless the positive calls using Wilcoxon signed rank test.

8.1.1 List of Tables

- Summary table of IgG response rate by analyte, visit time post vaccination, and regimen.
- Summary statistics for binding antibody responses AUC (minimum, 25th percentile, median, 75th percentile, maximum, mean, standard deviation) among all participants, by analyte, visit time post vaccination, and regimen.
- Summary statistics for binding antibody responses AUC (minimum, 25th percentile, median, 75th percentile, maximum, mean, standard deviation) among positive responders by analyte, visit month, and regimen.
- The comparisons of response rates and magnitudes between groups within visit and between time points within group as specified above.

8.1.2 List of Graphs

- Bar plots of IgG response rate and boxplots of magnitudes AUC by analyte and visit time. In one set of plots, the boxplots are built using data from positive responders only; in the second set of plots, boxplots are built using data from all participants regardless of response positivity calls.
- Spaghetti plots of responses over time by analyte, and regimen.

8.2 Circulating Immune Complex Panel Assay

Objective: To further evaluate the study participants' Adverse Events (urticaria) experienced post study product administration, the immune complexes will be analyzed in testing any associations with the grade 3 injection site erythema, induration, fever, and other AE of concern and compared to samples from participants who have not received the FP8v1-rTTHC (FP conjugate) vaccine.

The circulating immune complexes will be evaluated with ELISA on serum (SST) samples collected at the following time points:

- The first vaccination (Day 0), 1 week post the first vaccination (Day 7), and 2 weeks post the first vaccination (Day 14) from participants in Group 1,2, 5 in Part A and Groups 6-8 in Part B.
- 1 week post the 2nd vaccination (Day 35) and 2 weeks post the 2nd vaccination (Day 42) from participants in Groups 7-8 who received the 2nd vaccination.

First, we will assess whether the changes in the immune complexes (C1Q binding and C1C C3) at post vaccination visits from the baseline differ between those who had G3 AEs (including injection site erythema/redness, induration/swelling (either categorized as reactogenicity or as AE due to delayed-onset after reactogenicity period), fever, and serum sickness as "AE of concern") and those who didn't have any of those G3 AEs among the participants in Groups 1, 2, 7, and 8 who have received FP conjugate vaccine.

Secondly, we will assess whether the changes in the immune complexes (C1Q binding and C1C C3) at post vaccination visits from the baseline differ between groups with and without having G3 AEs among the participants in Groups 5 and 6 who haven't received FP conjugate vaccine.

Thirdly, we will assess whether the changes in the immune complexes (C1Q binding and C1C C3) at post vaccination visits from the baseline differ between groups with and without having G3 AEs among all participants who have and haven't received FP conjugate vaccine.

The following comparisons of the immune complexes will be done:

- Comparisons of C1Q binding and CIC C3 in fold change from the baseline visit (Day 0) between those who had G3 AEs and those who didn't have among participants in Groups 1-2, 7-8 who have received the 1st FP conjugate vaccine, at Day 7 and Day 14; among the participants in Groups 7-8 who have received the second FP conjugate vaccine, at Day 35 and 42; and among participants in Groups 5-6 who haven't received FP conjugate vaccine, at Day 7 and Day 14, using Wilcoxon rank sum test.
- Comparisons of C1Q binding and CIC C3 in fold change from the baseline visit between those who had and who didn't have G3 AEs among all participants, at Day 7 and Day 14, using Wilcoxon rank sum test.
- Comparisons of C1Q binding and CIC C3 between post vaccine visits vs the baseline visit by groups defined by with/without G3 AEs and received/not received FP conjugate vaccine using Wilcoxon signed rank test.
- Comparisons of C1Q binding and CIC C3 between post vaccine visits vs the baseline visit by groups defined by with/without G3 AEs using Wilcoxon signed rank test.
- Comparison of C1Q binding and CIC C3 at post vaccination visits between the 4 groups defined by with/without G3 AEs and received/not received FP conjugate vaccine jointly using the linear mixed model to account the correlation between visits within individuals and with the C1Q binding and CIC C3 at the baseline visit as covariates adjusted in the model. The group of the participants in Groups 5-6 who didn't have any of those G3 AEs is a reference group.
- Comparison of C1Q binding and CIC C3 at post vaccination visit between the 2 groups defined by with/without G3 AEs jointly using the linear mixed model to account the correlation between visits within individuals and with the C1Q binding and CIC C3 at the baseline visit as covariates adjusted in the model.

8.2.1 List of Tables

- Table of statistics summary of C1Q binding and CIC C3 by visit days and by the 4 groups defined by with/without G3 AEs and received/not received FP conjugate vaccine as well as 2 groups defined by with/without G3 AEs.
- Table of statistics summary of fold-change of C1Q binding and CIC C3 from the baseline by post vaccination visit days and by the 4 groups defined by with/without G3 AEs and received/not received FP conjugate vaccine as well as 2 groups defined by with/without G3 AEs.
- The comparisons of the immune complexes as specified above.
- List of participants who had a such G3 AE defined above (PUBID, the original group, AE event, maximum severity, vaccination #, days between the AE onset date and the vaccination date, immune complexes by visit).

8.2.2 List of Graphs

- Boxplots of the immune complexes (C1Q binding and CIC C3) by visit days with lines connecting observations from the same participants and by 4 groups defined by with/without G3 AEs and received/not received FP conjugate vaccine in different panels.
- Boxplots of the immune complexes (C1Q binding and CIC C3) by visit days with lines connecting observations from the same participants and by 2 groups defined by with/without G3 AEs in different panels.
- Boxplots of C1Q binding and CIC C3 in fold change from the baseline visit by post vac visit days with lines connecting observations from the same participants and by 4 groups defined with/without G3 AEs and received/not received FP conjugate vaccine in different panels.
- Boxplots of C1Q binding and CIC C3 in fold change from the baseline visit by post vac visit days with lines connecting observations from the same participants and by 2 groups defined with/without G3 AEs in different panels.
- Spaghetti (line) plots of the immune complexes (C1Q binding and CIC C3) over visit days by the original groups (G1-G2, G5- G6, G7-G8) (using different colors/symbols indicating whether having a such G3 AE).

8.3 MSD cytokine Assay

Serum cytokine concentrations were measured according to the manufacturer's instructions on several kits from Meso Scale Discovery.

The objectives/endpoints addressed are as follows:

- To evaluate any associations of the cytokines, TNF-α, IL-1β, IL-6, with clinical data on reactogenicity and adverse events observed in some participants in HVTN 303
- To evaluate any associations of the cytokines, IL-4, IL-5, IL-10, IL-13, IFN-γ with eosinophil values increases observed in some participants in HVTN 303
- To explore any associations of the rest cytokines (Eotaxin, Eotaxin-3, IL-8, IL-8 (HA), IP-10, MCP-1, MCP-4, MDC, MIP-1α, MIP-1β, TARC, IL-2, IL-8, IL-12p70, IgE, GM-CSF) with clinical data on reactogenicity and adverse events and eosinophil values increases observed in some participants in HVTN 303

The Meso Scale Discovery (MSD) electrochemiluminescent immunoassay platform was used for multiplex measurement of soluble immunomodulatory factors such as chemokines or cytokines. Serum

was tested according to the manufacturer's instructions on several kits from Meso Scale Discovery (Rockville, MD). Some kits were validated kits (V-Plex) while one was not (U-Plex).

Samples were not stimulated. Technical controls that were either provided as part of the V plex kits or purchased separately (QC-01, QC-02, and QC-03) of known concentrations were used. An in-house CMV stimulated PBMC supernatant control was used as the PBMC control.

MSD cytokine will be performed on all serum samples collected at baseline, 1 week post the first vaccination, 2 weeks post the first vaccination from participants in Groups 1-5 in Part A and Groups 6-8 in Part B, and 1 week post the 2nd vaccination, 2 weeks post the 2nd vaccination from participants in Groups 7-8 who received the 2nd vaccination.

Groups 1-5 (1 vaccination):

- V2 [D0, baseline]
- V3 [D7, 1 wk post 1 vacc]
- V4 [D14, 2 wk post 1 vacc]

Group 6 (1 vaccination):

- V101 [D0, baseline]
- V102 [D7, 1 wk post 1st vacc]
- V103 [D14, 2 wk post 1st vacc]

Group 7 (1 or 2 vaccinations):

- V101 [D0, baseline]
- V102 [D7, 1 wk post 1st vacc]
- V103 [D14, 2 wk post 1st vacc]
- V105 [D35, 1 wk post 2nd vacc]
- V106 [D42, 2 wk post 2nd vacc]

Group 8 (1 or 2 vaccinations):

- V101 [D0, baseline]
- V102 [D7, 1 wk post 1st vacc]
- V103 [D14, 2 wk post 1st vacc]
- V105 [D35, 1 wk post 2nd vacc]
- V106 [D42, 2 wk post 2nd vacc]

8.3.1 Cytokines analytes: TNF-α, IL-1β, and IL-6

Since all enrolled participants received at least one study vaccine with Adjuplex adjuvant, all enrolled participants from G1-G8 will be included in this analysis and categorized by whether they had G3 reactogenicity and AEs (including injection site erythema/redness, induration/swelling (either categorized as reactogenicity or as AE due to delayed-onset after reactogenicity period), fever, and serum sickness as "AE of concern"). We will assess any differences in the changes of TNF-α, IL-1β, and IL-6 at post vaccination visits from the baseline between participants who had G3 reactogenicity and AEs and those who didn't have any of those

G3 AEs. We will also assess differences in the changes of above cytokines at post vaccination visits from the baseline between groups with and without experiencing G3 AEs among participants who received 2 vaccinations.

8.3.1.1 List of Tables

- Tables of statistics summary of TNF-α, IL-1β, and IL-6 by visit days and by whether they had G3 reactogenicity and AEs
- Tables of statistics summary of TNF-α, IL-1β, and IL-6 by visit days and by whether they had G3 reactogenicity and AEs among participants who received 2 vaccinations
- Comparisons of TNF-α, IL-1β, and IL-6 between post vaccine visits vs the baseline visit within the groups defined by whether they had a G3 AE using Wilcoxon signed rank test
- Comparisons of TNF-α, IL-1β, and IL-6 between post vaccine visits vs the baseline visit within the groups defined by whether they had a G3 AE among participants who received 2 vaccinations using Wilcoxon signed rank test
- Comparisons of TNF-α, IL-1β, and IL-6 in fold change from the baseline visit between groups defined by whether they had a G3 AE within each post vac visit days using Wilcoxon rank sum test
- Comparisons of TNF-α, IL-1β, and IL-6 in fold change from the baseline visit between groups defined by whether they had a G3 AE within each post vac visit days among participants who received 2 vaccinations using Wilcoxon rank sum test
- Comparison of TNF-α, IL-1β, and IL-6 at post vaccination visits between the groups defined by whether they had a G3 AE using the linear mixed model to account the correlation between visits within individuals and adjusting the baseline values as a covariate
- Comparison of TNF-α, IL-1β, and IL-6 at post vaccination visits between the groups defined by whether they had a G3 AE among participants who received 2 vaccinations using the linear mixed model to account the correlation between visits within individuals and adjusting the baseline values as a covariate
- List of participants who had any G3 AEs (PUBID, AE event, maximum severity, vaccination #, days between the onset date and the vaccination date, TNF-α, IL-1β, and IL-6 values by visit)

8.3.1.2 List of Graphs

- Boxplots of TNF-α, IL-1β, and IL-6 by visit days and by whether they had G3 reactogenicity and AEs with lines connect observations from the same individuals
- Boxplots of TNF-α, IL-1β, and IL-6 by visit days and by whether they had G3 reactogenicity and AEs with lines connect observations from the same individuals among participants who received 2 vaccinations
- Boxplots of TNF-α, IL-1β, and IL-6 in fold change from the baseline visit by post vac visit days and by whether they had G3 reactogenicity and AEs
- Boxplots of TNF-α, IL-1β, and IL-6 in fold change from the baseline visit by post vac visit days and by whether they had G3 reactogenicity and AEs among participants who received 2 vaccinations
- Spaghetti (line) plots of TNF-α, IL-1β, and IL-6 over visit days (using different colors/symbols indicating whether having a such G3 AE).

8.3.2 Cytokines analytes: IL-4, IL-5, IL-10, IL-13, IFN-γ

In this analysis, all participants from G1-G8 will be included and grouped by whether they had an increased eosinophil values (eosinophil count ≥ 500 cells/mm3 or percentage eosinophil of WBC ≥ 5%)

at any of post vaccination visits. We will evaluate any differences in the cytokines (IL-4, IL-5, IL-10, IL-13, IFN-γ) between the participants who had an increased eosinophil values and who didn't and evaluate any correlations between the cytokines and eosinophil values (either in absolute counts or percentage of WBC) among the participants who had an increased eosinophil values and among the participants who didn't have an increased eosinophil values. We will also do the above analysis among participants who received 2 vaccinations.

8.3.2.1 List of Tables

- Tables of statistics summary of the cytokines and eosinophil by visit days and by whether they had increased eosinophil values
- Tables of statistics summary of the cytokines and eosinophil by visit days and by whether they had increased eosinophil values among participants who received 2 vaccinations
- Comparisons of the cytokines between post vaccine visits vs the baseline visit within the groups defined by whether they had increased eosinophil values using Wilcoxon signed rank test
- Comparisons of the cytokines between post vaccine visits vs the baseline visit within the groups defined by whether they had increased eosinophil values among participants who received 2 vaccinations using Wilcoxon signed rank test
- Comparisons of cytokines in fold change from the baseline visit between groups defined by whether they had increased eosinophil values within each post vac visit days using Wilcoxon rank sum test
- Comparisons of cytokines in fold change from the baseline visit between groups defined by whether they had increased eosinophil values within each post vac visit days among participants who received 2 vaccinations using Wilcoxon rank sum test
- Comparison of cytokines at post vaccination visits between the groups defined by whether they had increased eosinophil values using the linear mixed model to account the correlation between visits within individuals and adjusting the baseline values as a covariate
- Comparison of cytokines at post vaccination visits between the groups defined by whether they had increased eosinophil values among participants who received 2 vaccinations using the linear mixed model to account the correlation between visits within individuals and adjusting the baseline values as a covariate
- List of participants who had increased eosinophil values (PUBID, # of vaccinations, the cytokines and eosinophil values by visit)

8.3.2.2 List of Graphs

- Boxplots of the cytokines by visit days and by whether had increased eosinophil values with lines connect observations from the same individuals
- Boxplots of the cytokines by visit days and by whether had increased eosinophil values with lines connect observations from the same individuals among participants who received 2 vaccinations
- Boxplots of the cytokines and in fold change from the baseline visit by post vac visit days and by whether they had increased eosinophil values
- Boxplots of the cytokines and eosinophil in fold change from the baseline visit by post vac visit days and by whether they had increased eosinophil values among participants who received 2 vaccinations
- The scatterplot of the cytokines vs eosinophil values by visit days with color-coded for participants who had and didn't have increased eosinophil values and the regression line for each group defined by whether they had increased eosinophil values with Spearman

correlations between the cytokines and eosinophil values by visit among the participants who had increased eosinophil values and among the participants who did not have

- The scatterplot of the cytokines vs eosinophil values by visit days with color-coded for participants who had and didn't have increased eosinophil values and the regression line for each group defined by whether they had increased eosinophil values among participants who received 2 vaccinations with Spearman correlations between the cytokines and eosinophil values by visit among the participants who had increased eosinophil values and among the participants who did not have
- Spaghetti (line) plots of IL-4, IL-5, IL-10, IL-13, IFN-γ over visit days (using different colors/symbols indicating whether they had increased eosinophil values)
- Spaghetti (line) plots of IL-4, IL-5, IL-10, IL-13, IFN-γ over visit days (using different colors/symbols indicating whether they had increased eosinophil values) among participants who received 2 vaccinations
- Spaghetti (line) plots of the eosinophil by visit days and by whether had increased eosinophil values with lines connect observations from the same individuals
- Spaghetti (line) of the eosinophil by visit days and by whether had increased eosinophil values with lines connect observations from the same individuals among participants who received 2 vaccinations
- Spaghetti (line) plots of the percentage eosinophil of WBC by visit days and by whether had increased eosinophil values with lines connect observations from the same individuals
- Spaghetti (line) of the percentage eosinophil of WBC by visit days and by whether had increased eosinophil values with lines connect observations from the same individuals among participants who received 2 vaccinations

8.4 Neutralizing Antibody

The purpose of this assay is to address the exploratory objective I:

- Mapping of FP-specific serum neutralizing activity.

Neutralization assay will be using pseudoviruses matched to vaccine strains, along with mutants thereof and other strains that are highly sensitive to FP antibodies. Neutralizing antibodies against HIV-1 will be measured as a function of reductions in Tat-regulated luciferase (Luc) reporter gene expression in TZM-bl cells at 2 weeks post the first vaccination from participants in Group 1-5 in Part A and Groups 6-8 in Part B, and 2 weeks post the 2nd vaccination from participants in Groups 7-8 who received the 2nd vaccination.

Part A (Groups 1-5, 1 vaccination):

- V4 [W2, 2 wk post 1st vacc]

Part B (Group 6, 1 vaccination):

- V103 [W2, 2 wk post 1st vacc]

Part B (Groups 7-8, 1 or 2 vaccinations):

- V103 [W2, 2 wk post 1st vacc]
- V106 [W6, 2 wk post 2nd vacc]

The assay will be performed in TZM-bl cells measured neutralization titers against a panel of autologous and/or heterologous Env-pseudotyped viruses that exhibit the following naturalization phenotypes:

- Env-pseudotyped viruses that are sensitive to fusion peptide nNAb neutralization and exhibit a tier 2 neutralization phenotype grown in 293T/17 cells:
    - H704_1528_240_RE_pblib_001_s
    - H704_1180_070EsN
    - H704_1835_150_RE_p001s_2484A
    - H704_0907_130sN
    - BG505
    - BG505.N611Q
    - SIV

Data are reported as ID50 or ID80 – reciprocal serum dilution required to achieve 50% or 80% neutralization, respectively – calculated using a dose-response curve-fit formula integrated in the VHICL-IMS (VRC Humoral Immunology Core Laboratory Information System). Response to a virus/isolate was considered positive if the neutralization titer was equal or above a pre-specified cutoff. A titer was defined as the serum dilution that reduced relative luminescence units (RLUs) by 50% relative to the RLUs in virus control wells (cells + virus only) after subtraction of background RLU (cells only). The prespecified cutoff was 20 for TZM-bl cells.

Tables show the response rates to each virus/isolate and corresponding 95% confidence intervals calculated by the Wilson score test method, as well as summary statistics among positive responders and both responders and non-responders. Plots of neutralizing antibody titers show both response rates and the distribution of magnitude among positive responders. Positive responses are indicated by dots color-coded by treatment group, and negative responses by gray triangles. Data points for each participant are connected by a gray line. The mid-line of the box denotes the median and the ends of the box denote the 25th and 75th percentiles. The whiskers that extend from the top and bottom of the box extend to the most extreme data points that are no more than 1.5 times the interquartile range (i.e., height of the box) or if no value meets this criterion, to the data extremes.

8.4.1 List of Tables

- Response rate table by virus, visit day, and treatment group.
- Summary statistics (i.e., min, mean, median, max) among positive responders by virus, visit day, and treatment group.
- Summary statistics (i.e., min, mean, median, max) among all participants (positive and negative responders) by virus, visit day, and treatment group.

8.4.2 List of Graphs

- Boxplots of neutralizing antibody titers by virus, and treatment group for T1-T6.
- Boxplots of neutralizing antibody titers by virus, visit day, and treatment group for T7-T8.

8.5 B-Cell Phenotyping

The purpose of this assay is to address the exploratory objective 4:

- To conduct analyses related to furthering the understanding of HIV, immunology, vaccines, and clinical trial conduct. To further evaluate immunogenicity of each vaccine regimen, additional immunogenicity assays may be performed in a subset of participants, including on samples from other timepoints, based on the HVTN Laboratory Assay Algorithm.

Frozen PBMC samples will be negatively enriched for B cells before staining with a high-parameter flow cytometry panel containing vaccine-specific B cell probes. Stained cells will be analyzed by flow cytometry, using a BD S6 sorter, while simultaneously selecting antigen-specific B cells of interest for cell sorting in preparation for B cell repertoire analysis. Analysis will include determining the frequency of vaccine-specific B cells using the FP, Trimer 4571 and Trimer 6931 probes.

To prepare the samples for flow cytometry analysis, cryopreserved PBMCs were thawed and transferred to 9ml of pre-warmed RPMI supplemented with 10% FBS and 2µl of Benzonase in a 15ml tube. The cells were then centrifuged at 1500 rpm for 5 minutes, and the supernatant was discarded. Any residual media was removed by gently blotting the tube on a Kimwipe. Subsequently, the cells were resuspended in 2ml of 1X PBS and transferred to a 5ml round-bottom tube. After centrifugation at 1500 rpm for 5 minutes, the supernatant was discarded, and any residual PBS was removed by blotting. The cells were then resuspended in 50µl of LIVE/DEAD fixable Aqua stain and incubated for 2 minutes at room temperature in the dark. Next, 100µl of surface antibody mix, which included the FP, Trimer 4571, and Trimer 6931 probes diluted in BD Brilliant Stain Buffer, was added to each tube. The samples were mixed by pipetting and incubated for 30 minutes at 4°C in the dark. After incubation, the cells were washed twice by adding 2ml of 1x PBS containing 0.1% BSA to each tube, followed by centrifugation at 1500 rpm for 5 minutes. The supernatant was discarded, and any residual PBS was removed by blotting. This washing step was repeated twice. Finally, the cells were resuspended in 200µl of cold RPMI containing 10% FBS and immediately prepared for sorting.

Samples that are tested for BCP were collected at baseline (V101, W0) and 2 weeks post 2nd vaccination (V106, W6) in Groups 7-8 in Part B who received the 2nd vaccination.

Response positivity was assessed for each B cell subset combination of interest, and for each participant sample collected post-vaccination. Refer to the table below for combinations presented in this report.

Label	Numerator gate	Denominator gate
%Trimer4571+ of IgG	Trimer4571+	IgG
%Trimer4571+Trimer 6931+ of IgG	Trimer4571+Trimer 6931+	IgG

%Trimer4571+Trimer 6931+FP+ of IgG	Trimer4571+Trimer 6931+FP+	IgG
%Trimer4571+ FP+ of IgG	Trimer4571+ FP+	IgG
%FP+ of IgG	FP+	IgG
%Trimer 6931+FP+ of IgG	Trimer 6931+FP+	IgG
%Trimer 6931+ of IgG	Trimer 6931+	IgG
%Trimer4571+ of B cells	Trimer4571+	B cells
%Trimer4571+Trimer 6931+ of B cells	Trimer4571+Trimer 6931+	B cells
%Trimer4571+Trimer 6931+FP+ of B cells	Trimer4571+Trimer 6931+FP+	B cells
%Trimer4571+ FP+ of B cells	Trimer4571+ FP+	B cells
%FP+ of B cells	FP+	B cells
%Trimer 6931+FP+ of B cells	Trimer 6931+FP+	B cells
%Trimer 6931+ of B cells	Trimer 6931+	B cells

To assess positivity for the detection of vaccine-specific B cells and IgG B cells, a Fisher's exact test was used: A two-by-two contingency table was constructed comparing the post-vaccination and baseline (Visit 101) data. The four entries in each table were (1) the number of Env-specific B cells over the number of B cells after vaccination (2) the number of B cells which were not Env-specific after vaccination and (3) the number of Env-specific B cells over the number of B cells at baseline (4) the number of B cells which were not Env-specific at baseline. A one-sided Fisher's exact test was applied to the table, testing whether the percent of Env-specific B cells for the post-vaccination data was equal to that for the data from baseline, versus an alternative hypothesis that it is greater. Because the sample sizes (i.e., total cell counts for the B cell subset) were 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 p-value significance threshold was chosen stringently ($\leq$0.00001).

Tables show the response rates and corresponding 95% confidence intervals calculated by the score test method [1]. Barnard's exact test was used to compare response rates between treatment

groups, while the Wilcoxon rank sum test was used to compare response magnitudes between treatment groups, among positive and negative responders, and among the subset of positive responder. McNemar's test was used to compare response rates between visits within treatment group, while Wilcoxon signed rank test was used to compare response magnitudes between visits within treatment group among all participants and among positive responders.

The distribution of the frequency of B cell response is displayed graphically on the log scale by B cell subset, visit, and treatment groups. The y-axis is truncated at 0.025% and any values below this level are censored. Plots include data from responders in color and non-responders in gray. Data points over time for each participant are connected by a gray line. Box plots based upon data from responders only are superimposed on the distributions. The mid-line of the box denotes the median and the ends of the box denote the 25th and 75th percentiles. The whiskers that extend from the top and bottom of the box extend to the most extreme data points that are no more than 1.5 times the interquartile range (i.e., height of the box) or if no value meets this criterion, to the data extremes.

8.5.1 List of Tables

- Response rate table by B cell subset, visit day, and treatment group.
- Summary statistics (minimum, 25th percentile, median, 75th percentile, maximum, mean, standard deviation) among all participants, by B cell subset, visit day, and treatment group.
- Summary statistics (minimum, 25th percentile, median, 75th percentile, maximum, mean, standard deviation) among positive responders by B cell subset, visit day, and treatment group.
- Response rates comparisons of Group 7 vs Group 8 using Barnard's exact test
- Response magnitude comparisons of Group 7 vs Group 8 using Wilcoxon rank sum test
- Response rates comparison between visits within treatment group using McNemar's test
- Response magnitude comparison between visits within treatment group among all participants using Wilcoxon signed rank test
- Response magnitude comparison between visits within treatment group among positive responders using Wilcoxon signed rank test

8.5.2 List of Graphs

- Boxplots of % B cell subset over time by treatment group.

8.6 Microscopy-Based Polyclonal Epitope Mapping (EMPEM)

The purpose of this assay is to address the exploratory objective 3:

- To evaluate the humoral and cellular immune response to vaccination regimens including FP-conjugate vaccine and Trimer 4571 to compare responses between the regimens.

Electron Microscopy Polyclonal Epitope Mapping (EMPEM) will be used to assess epitopes of serum polyclonal Ab (Fab) binding to HIV-1 Env in samples collected at V107 [W8, 4 weeks post 2nd vaccination], V108 [W9, 5 weeks post 2nd vaccination], or V109 [W10, 6 weeks post 2nd vaccination] from participants in Groups 7 and 8 who received the 2nd vaccination. EMPEM is

amenable to trimeric Env immunogens (e.g., Trimer 4571). The FP-conjugate immunogen is too small in mass to perform accurate mapping by EM. Therefore, only samples obtained after Trimer 4571 boost will be analyzed by EMPEM, using Trimer 4571 as the probe. The antigen (Trimer 4571) provided by VRC has undergone negative-stain electron microscopy quality control for each lot used in this study. This process ensures that the antigen is trimeric and native-like prior to incubation with polyclonal Fab.

Polyclonal antibodies (IgG) are isolated from individual (participant) blood samples using commercial Fc-affinity purification resins. The antibodies are enzymatically digested into the fragment antigen binding (Fab) components with papain, and incubated with soluble, HIV Env trimer proteins, matched to the immunogen used in the study (Trimer 4571). The complex is purified by size-exclusion chromatography, adsorbed onto electron microscopy (EM) grids, stained, and imaged. Individual protein complex particles are extracted from the images and subjected to averaging and classification in 2D and 3D space. 3D focused classification methods are used to evaluate each of the defined epitopes on the surface of Env, both in qualitative and quantitative (the magnitude of the given epitope response) terms. The end results are 3D EM maps that are matched to known structures of Env in complex with antibodies and each polyclonal Fab specificity is assigned a final epitope label based on overlap with known structure(s).

The non-quantitative results are graphical and illustrative representations of all unique Env epitopes detected, as a function of individual and timepoint. The quantitative results are per-epitope EMPEM magnitudes, calculated as a function of the number of particles containing a given polyclonal Fab relative to the total number of particles evaluated in 3D space. The per-epitope magnitude value is on a scale of 0-3 to account for trimeric Env having up to 3 copies of a given epitope. It is analogous to stoichiometry (i.e. a value of 3 suggests that all particles had all three copies of the same epitope fully occupied by polyclonal Fab). Note that protomers are an observed phenotype (trimer dissociation/disassembly) and not a unique antibody. These epitopes cannot be accurately mapped and as such have only non-quantitative results. The assay is further described in Bianchi et al. (2018) [9], Turner et al. (2023) [10], and Hahn et al. (2024) [11].

The epitopes assignments are: gp41-base, V1V2V3, gp41-GH, C3V5, CD4bs, gp120-GH, gp120-gp120, and gp41-FP (GH: glycan hole, FP: fusion peptide, CD4bs: CD4 binding site). If antibody induced trimer disassembly is detected during 2D classification, the sample is also assigned a label: protomers.

Examples of EMPEM-derived 3D maps and the location of each epitope with respect to HIV Env ectodomain are shown in Figure 1.

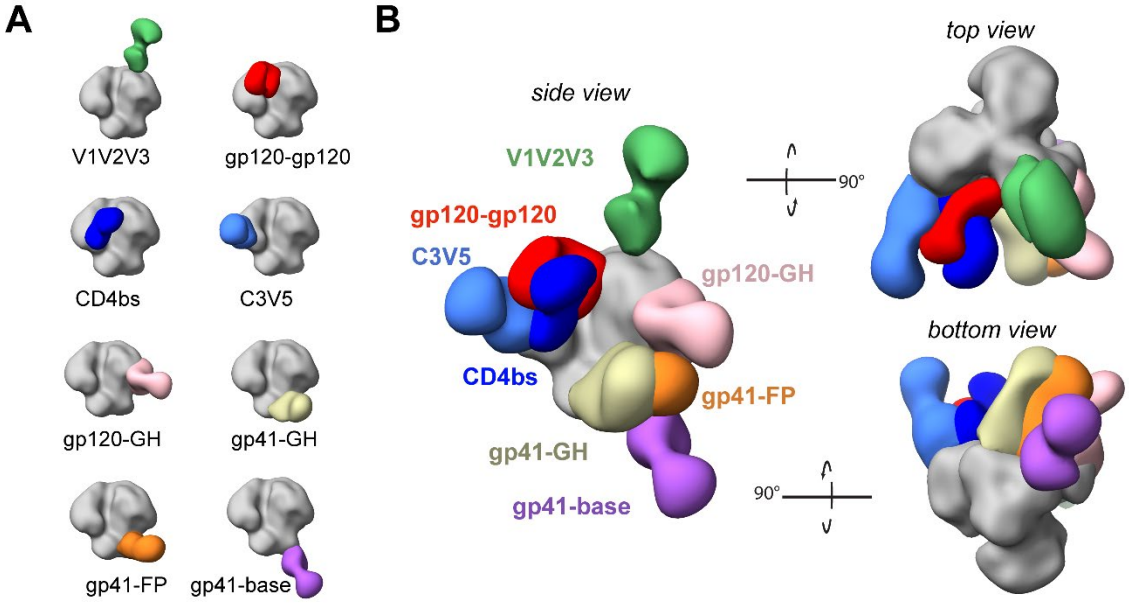

Figure 1. Visualization of EMPEM-derived 3D maps. A) Representative maps of antibody-trimer complexes for all defined EMPEM epitopes. B) Overlay of antibodies to the 8 defined EMPEM epitopes.

For each sample and epitope, response was considered positive if epitope mapping was successful. The total magnitude, with or without including the gp41-base, is calculated as the sum of positive response epitope magnitudes.

Plots of magnitude show both response rates and the distribution of magnitude. Positive responses are indicated by dots color-coded by treatment group, and negative responses by gray triangles. The mid-line of the box denotes the median and the ends of the box denote the 25th and 75th percentiles. The whiskers that extend from the top and bottom of the box extend to the most extreme data points that are no more than 1.5 times the interquartile range (i.e., height of the box) or if no value meets this criterion, to the data extremes.

Tables show the response rates and corresponding 95% confidence intervals calculated by the score test method [1]. Summary statistics of response magnitudes were tabulated for all participants and the subset of positive responders by antigen, epitope, treatment group, and visit day.

8.6.1 List of Tables

- Response rate table by antigen, epitope, and visit day.
- Summary statistics of response magnitudes (minimum, 25th percentile, median, 75th percentile, maximum, mean, standard deviation) among all participants, by antigen, epitope, and visit day.
- Summary statistics of response magnitudes (minimum, 25th percentile, median, 75th percentile, maximum, mean, standard deviation) among positive responders by antigen, epitope, and visit day.

8.6.2 List of Graphs

- Box and bar plots by epitope show both response rates and the distribution of magnitude where the boxplot is drawn around only positive responders.
- Bar plots illustrate the percentage of participants with protomers detected.
- Box and bar plots by the total magnitude, with or without including the gp41-base show both response rates and the distribution of magnitude where the boxplot is drawn around only positive responders.
- A participant-level plot shows which epitopes were positive for each participant and treatment group.

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