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STATISTICAL ANALYSIS PLAN FOR HVTN IMMUNOGENICITY

Protocol HVTN 304 (v4.0)

A phase 1 open-label clinical trial to evaluate the safety and immunogenicity of synthetic DNAs encoding a native-like HIV Env Trimer and Interleukin-12 (INO-6160), alone or in a prime-boost regimen with 3M-052-AF + Alum adjuvanted VRC HIV Env Trimer 4571 in adult participants without HIV

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Statistical Analysis Plan for Immunogenicity

Protocol: HVTN 304 (2.0)

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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	Added BCP assay (section 8.3)
3.0	Added ADCC, ICABA, BAMA, and EMPEM assays (sections 8.4-8.7)
4.0	Added differential binding tables and figures to BAMA (section 8.6) Updated BCP markers and removed comparison tables (section 8.3)

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1 OVERVIEW

The following describes the Statistical Analysis Plan (SAP) for the analysis of immunogenicity data from HVTN 304 for Protocol Team (PT) reports of immunogenicity data, and for the Final Study Report (FSR) of Immunogenicity.

2 PROTOCOL SUMMARY

Title

A phase 1 open-label clinical trial to evaluate the safety and immunogenicity of synthetic DNAs encoding a native-like HIV Env Trimer and Interleukin-12 (INO-6160), alone or in a prime-boost regimen with 3M-052-AF + Alum adjuvanted VRC HIV Env Trimer 4571 in adult participants without HIV.

Design

This is a randomized open-label trial to examine the safety and immunogenicity of INO-6160 (synthetic DNAs encoding a native-like HIV Env Trimer and Interleukin-12), alone or in a prime-boost regimen with VRC HIV Env Trimer 4571 adjuvanted with 3M-052-AF + Alum. The primary hypothesis is that the vaccine regimen will elicit HIV-1 envelope protein–specific binding antibody (Ab) and T-cell responses.

Study products, diluents, and electroporation device

• INO-6160: sD-NLT-AB05 co-formulated with IL-12 DNA (pGX6001): sD-NLT-AB05 consists of a single plasmid, pGX1060 (in pGX0001 vector backbone), encoding a soluble stabilized native-like trimer derived from clade A isolate BG505. pGX6001 (pGX0003 vector backbone), contains a dual promoter system for expression of both the human IL-12 p35 and p40 genes necessary for production of the active heterodimeric IL-12 protein. The plasmid ratio for the coformulated drug product is 4:1 (0.8 mg pGX1060/0.2 mg pGX6001) per 0.1 mL/1 mg injection. The coformulation, INO-6160, in water-for-injection (WFI), is supplied at a concentration of 10 mg/mL and a volume of 0.4 mL in 2-mL glass vials.

• Trimer 4571: HIV-1 Env Trimer 4571 (VRC-HIVRG096-00-VP) is a soluble protein that consists of BG505 DS-SOSIP.664 gp140 Env and is supplied as a sterile, aqueous, buffered solution filled into single-dose vials at a concentration of 500 mcg/mL and a volume of 1.2 mL in 3-mL glass vials. Trimer 4571 is provided by the Dale and Betty Bumpers Vaccine Research Center (VRC) and will be used at a dose of 100 mcg.

• 3M-052-AF adjuvant: This adjuvant is an aqueous formulation (AF) of the small molecule imidazoquinoline, which acts as a toll-like receptor (TLR) 7/8 agonist. 3M-052-AF is supplied at a concentration of 50 mcg/mL and a fill volume of 0.4 mL in 2-mL glass vials.

• Aluminum Hydroxide Suspension, Adjuvant: Aluminum hydroxide suspension (Alum) is composed of Alhydrogel 2% (Brenntag Biosector, Frederikssund, Denmark) diluted with WFI to a concentration of 5 mg/mL. It is supplied as a sterile, pyrogen-free suspension filled into single-dose vials at a volume of 0.7 mL.

• Electroporation device: The Inovio CELLECTRA Adaptive Constant Current Electroporation (EP) Device is a portable, battery-powered medical device designed to facilitate the introduction

of DNA into skin through EP. The Inovio CELLECTRA 2000 will be used for intradermal (ID) delivery following Mantoux injection of the DNA vaccine and is provided by Inovio Pharmaceuticals.

Study participants

20 healthy volunteers without HIV, 18 through 55 years of age in the United States.

Study plan and schema table

Participants will be evaluated for safety and immune responses through blood collection at specified timepoints throughout the study. The study schema is below:

				Injection Schedule			
Group	**N**	**Product/Dose**	**Route**	**Month 0**	**Month 1**	**Month 3**	**Month 6**
1	10	INO-6160 / 2.0 mg	ID EP	X	X	X	X
2	10	INO-6160 / 2.0 mg	ID EP	X	X	X	X
		Trimer-4571 / 100 mcg 3M-052-AF (5 mcg) + Alum (500 mcg)	IM	--	--	X	X
Total	**20***						

Enrollment was restricted to 1 participant per day for the first 5 participants (across both arms) and enrollment paused after the first 5 participants were enrolled. The Protocol Safety Review Team (PSRT)reviewed cumulative safety information for all participants recorded through the visit scheduled 2 weeks post first vaccination for the first 5 participants and determined it was safe to proceed with full enrollment. The full enrollment was completed as of July 7, 2023 with a total of 20 participants.

Duration per participant

12 months of scheduled clinic visits (main study) and an AESI health contact at month 18.

Estimated total study duration

24 months (includes enrollment, planned safety holds, follow-up, and AESI health contact).

Study sites

The HIV Vaccine Trials Network (HVTN) Clinical Research Sites (CRSs) will be located in the US and will be further specified in the Site Announcement Memo.

3 OBJECTIVES AND ENDPOINTS

3.1 Primary objectives and endpoints

Objectives **Endpoints**

1. To evaluate the safety and tolerability of 2 doses of sD-NLT-AB05 + IL-12 DNA adjuvant followed by 2 doses of sD-NLT-AB05 + IL-12 DNA adjuvant alone or in combination with Trimer 4571 adjuvanted with 3M-052-AF + Alum	a) Local and systemic reactogenicity signs and symptoms will be collected for a minimum of 2 weeks following receipt of any study vaccine b) 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 will be collected throughout the study and for 12 months following any receipt of study product. Additionally, all adverse events will be collected for 30 days after any receipt of study vaccination.
2. To evaluate the immunogenicity of 2 doses of sD-NLT-AB05 + IL-12 DNA adjuvant followed by 2 doses of sD-NLT-AB05 + IL-12 DNA adjuvant alone or in combination with Trimer 4571 adjuvanted with 3M-052-AF + Alum	a) Response rate and magnitude of vaccine-matched IgG binding Ab responses as assessed by multiplex assay 2 weeks following the fourth vaccination b) Response rate and magnitude of CD4 + and CD8+ T-cell responses measured by flow cytometry, to HIV-1-specific Env peptide pools, 2 weeks following the fourth vaccination

3.2 Secondary objectives and endpoints

Objectives	Endpoints
1. To further evaluate and compare the ability of 2 doses of sD-NLT-AB05 + IL-12 DNA adjuvant followed by 1 or 2 doses of sD-NLT-AB05 + IL-12 DNA adjuvant alone or in combination with Trimer 4571 adjuvanted with 3M-052-AF + Alum to elicit humoral immune responses	a) Neutralizing Ab magnitude and breadth against autologous and tier 1a HIV-1 isolates as assessed by TZM-bl neutralization assay following the third and fourth vaccinations b) Response rate, magnitude, and epitope specificity of HIV-1 specific IgG binding Ab responses as assessed by multiplex assay 2 weeks following third vaccination
2. To further evaluate and compare the ability of 2 doses of sD-NLT-AB05 + IL-12 DNA adjuvant alone or in combination with Trimer 4571 adjuvanted with 3M-052-AF + Alum to elicit cellular immune responses	a) Response rate and magnitude of CD4 + and CD8+ T-cell responses measured by flow cytometry, to HIV-1-specific Env peptide pools, 2 weeks following third vaccination
3. To evaluate the durability of cellular and humoral immune responses elicited by 2 doses of sD-NLT-AB05 + IL-12 DNA adjuvant followed by 2 doses of sD-NLT-AB05 + IL-12 DNA adjuvant alone or in combination with Trimer 4571 adjuvanted with 3M-052-AF + Alum	a) Magnitude and response rate of CD4 + and CD8+ T-cell responses measured by flow cytometry, to HIV-1-specific Env peptide pools 6 months post last vaccination b) Response rate and magnitude of HIV-1 specific IgG binding Ab responses as assessed by multiplex assay 6 months post last vaccination c) Neutralizing Ab magnitude and breadth against autologous tier 2 HIV-1 isolates as assessed by TZM-bl neutralization assay 6 months post last vaccination

3.3 Exploratory Objectives

1. To clinically evaluate EP-injection–related skin changes for 6 months after the last study product administration and subjective assessment by participant of tolerability at 12 months after the last study product administration.

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2. To evaluate the response rate and magnitude of HIV-1 specific IgG binding Ab responses as assessed by multiplex assay 2 weeks following second vaccination.

3. To evaluate the response rate and magnitude of CD4 + and CD8+ T-cell responses measured by flow cytometry, to HIV-1-specific Env peptide pools, 2 weeks following the second vaccination.

4. To evaluate the frequency of Env-specific B cells measured by flow cytometry 2 weeks following the second, third, and fourth vaccinations and 6 months post last vaccination.

5. To evaluate serum Ab specificities and elicitation of trimer-degrading Abs using polyclonal epitope mapping as assessed by Electron Microscopy 2 weeks post third and fourth vaccinations.

6. To evaluate the neutralizing Ab magnitude and breadth against heterologous and tier 2 HIV-1 isolates as assessed by TZM-bl neutralization assay following the third, and fourth vaccinations.

7. To evaluate of HIV-1 specific IgG binding Ab responses to the trimer base 2 weeks following second, third, and fourth vaccinations.

8. To evaluate Ab avidity and Fc Receptor functions such as FcR binding, ADCC, antibody-dependent cellular phagocytosis (ADCP), and infected cell antibody-binding assay (ICABA) after the second, third, and fourth vaccinations.

9. To evaluate B-cell receptor (BCR) repertoires and sequences (including analysis of rare B-cell lineages associated with bnAb precursors), including cellular phenotyping and mutational frequency analysis suggestive of somatic hypermutation and affinity maturation following immunization.

10. To characterize monoclonal Abs derived from BCR sequences from Env-specific B cells.

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

12. 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 Portfolio.

4 COHORT DEFINITION

A total of 20 participants were enrolled in the study, 10 in Group 1 and 10 in Group 2.

5 RANDOMIZATION

The randomization sequence will be obtained by computer-generated random numbers and provided to each HVTN CRS through the Statistics and Data Management Center's (SDMC) Web-based randomization system. The randomization will be done in blocks to ensure balance across study groups. At each institution, the pharmacist with primary responsibility for dispensing study products is charged with maintaining security of the treatment assignments.

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6 BLINDING

This is an open-label study. Participants and site staff will be unblinded to participants' group assignments. Laboratory program staff will be blinded to participants' group assignments during assay analysis, whenever feasible.

7 STATISTICAL ANALYSIS

All data from enrolled participants will be analyzed according to the initial randomization assignment regardless of how many vaccinations they received. The analysis is a modified intent-to-treat analysis in that individuals who are randomized but not enrolled do not contribute data and hence are excluded. Because of the brief length of time between randomization and enrollment—typically no more than 4 working days—very few such individuals are expected. All analyses will be performed using SAS and R. Supporting analyses will be performed restricting to subsets of participants who received the complete series of vaccinations.

No formal multiple comparison adjustments will be employed for multiple safety endpoints, multiple primary immunogenicity endpoints, or secondary endpoints. However, multiplicity adjustments will be made for certain immunogenicity assays, as discussed below, when the assay endpoint is viewed as a collection of hypotheses (eg, testing multiple peptide pools to determine a positive response).

7.1 Analysis Variables

The analysis variables consist of baseline participant characteristic, and immunogenicity for primary- and secondary-objective analyses.

7.2 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, from specimens collected outside of the visit window, or from HIV-infected participants are excluded. Since the exact date of HIV infection is unknown, any assay data from blood draws 4 weeks, or less, 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 tests will be used to compare the response rates between 2 vaccine groups at a given time-point, McNemar's tests will be used for paired data (between visits), with a significant difference declared if the 2-sided p-value is ≤ 0.05.

For quantitative assay data (eg, magnitude of HIV-1 Env-specific binding Ab responses), graphical and tabular summaries of the distributions by antigen, treatment arm, and timepoint will be made. The difference between arms at a specific timepoint will be tested with a nonparametric Wilcoxon rank sum test and the difference between visits will be tested with a nonparametric Wilcoxon signed rank test including all available data regardless of response positivity. Response magnitudes below the limit of detection of the assay will be replaced by half of the limit in the

tests. All statistical tests will be 2-sided and will be considered statistically significant if $p \leq 0.05$.

7.3 Missing Data

Based upon previous AIDS Vaccine Evaluation Group and HVTN trials, missing 10% 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 peripheral blood mononuclear cells (PBMCs). Due to the limited sample size of the study, nonparametric tests as mentioned in the previous section will be used assuming the probability of an observation being missing does not depend upon any unobserved covariates and violations of this assumption will have little impact on the estimates and hypothesis tests.

7.4 Analyses prior to end of scheduled follow-up visits

Any analyses conducted prior to the end of the scheduled follow-up visits should not compromise the integrity of the trial in terms of participant retention or safety or immunogenicity endpoint assessments. In particular, early analyses by treatment assignment require careful consideration and should be made available on a need-to-know basis only.

A statistical analysis by treatment assignment of a primary immunogenicity endpoint may be performed. The HVTN Laboratory Program will review the analysis report prior to distribution to the protocol chairs, Division of AIDS (DAIDS), vaccine developer, and other key HVTN members and investigators. Distribution of reports will be limited to those with a need to know for the purpose of informing future trial-related decisions. The HVTN leadership must approve any other requests for HVTN immunogenicity analyses prior to the end of the scheduled follow-up visits. Any analyses conducted prior to the end of the study should not compromise the integrity of the trial in terms of participant retention or safety or immunogenicity endpoint assessments.

8 IMMUNOGENICITY TABLES AND FIGURES, BY ASSAY

If any participants missed the IM administration at Month 3 in Group 2, the analyses of immunogenicity data collected at Visit 7 (2 weeks after the 3rd vaccination) will be done for all participants, and for the subset of participants who received the full Month 0, Month 1 and Month 3 vaccinations in both study groups. If any participants missed the IM administration at Month 6 in Group 2, the analyses of immunogenicity data collected at Visit 9 (2 weeks after the 3rd vaccination) and Visit 10 (6 months after the 4th vaccination) will be done for all participants, and for the subset of participants who received the full Month 0, Month 1, Month 3 and Month 6 vaccinations in both study groups.

8.1 Intracellular Cytokine Staining

Flow cytometry is employed to examine HIV-1-specific CD4+ and CD8+ T-cell responses using a validated ICS assay. The vaccine-matched peptide pools evaluated are:

- BG505 gp120
- BG505 gp41
- A-AB05

Previously cryopreserved specimens are stimulated with the synthetic peptide pools. As a negative control, cells are left unstimulated. As a positive control, cells are stimulated with a polyclonal stimulant, staphylococcal enterotoxin B (SEB). There are no replicates except for the

negative control, which has two replicates. As an additional internal control, cells are stimulated with a CMVpp65 peptide pool.

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

To assess positivity for a peptide pool within a T-cell subset, a two-by-two contingency table is 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 IL-2, for both the stimulated and the negative control data. If both negative control replicates are included, then the sum number of total cells and the sum number of positive cells is 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 considered, 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.

Initially, PBMC samples from the following timepoints will be tested.

- Visit 7 (2 weeks after the 3rd vaccination) (PT Report #1)
- Visit 9 (2 weeks after the 4th vaccination) (PT Report #2)
- Visit 10 (6 months after the 4th vaccination) (PT Report #3)

Visit 5 (2 weeks after the 2nd vaccination) may be assayed contingent on responses from other timepoints and from other immune assays such as BAMA. Additional timepoints may be tested in the future.

8.1.1 List of Tables

- Response rate table by lab, T-cell subset, cytokine, peptide pool, visit, day, and treatment arm
- Summary statistics (i.e., min, mean, median, max) among responders for T-cell subset, peptide pool, visit, and treatment arm
- Summary statistics (i.e., min, mean, median, max) among all participants (positive and negative responders) for T-cell subset, peptide pool, visit, and treatment arm
- Response rate and/or response magnitude comparison of treatment arms by T-cell subset, cytokine, and visit.

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8.1.2 List of Graphs

- Boxplots of background-adjusted IFN-γ and/or IL-2 response magnitude by T-cell subset, HIV protein, visit, and treatment arm. [*Treatment groups side by side per protein, one T-cell subset and one visit per graph).*]
- For PT reports #2 and #3 when data from multiple visits are available, Spaghetti plots background-adjusted IFN-γ and/or IL-2 response magnitude by T-cell subset, HIV protein, visit, and treatment arm. [*Treatment groups side by side per protein, one T-cell subset and one visit per graph).*]

8.2 Neutralizing Antibody

Neutralization assays will measure titers in TZM-bl cells against BG505/T332N and MW965.26 with samples from: Baseline (visit 1), 2 weeks post 3rd dose (visit 7), 2 weeks post 4th dose (visit 9), and 6 months post 4th dose (visit 10). Positivity calls will be an unrounded ID50 value ≥10, the starting dilution, which is the limit of detection of the assay. Any samples resulting in >100 ID50 titer against BG505/T332N will be tested against a 12-virus panel used to assess heterologous tier 2 neutralization grown in 293T/17 cells.

8.2.1 List of Tables

- Response rate table by lab, assay type, virus, visit, day, and treatment arm
- Summary statistics (i.e., min, mean, median, max) among responders by lab, assay type, virus, visit, day, and treatment arm
- Summary statistics (i.e., min, mean, median, max) among all participants (positive and negative responders) by lab, assay type, virus, visit, day, and treatment arm
- Response rate and/or response magnitude comparison of treatment arms by visit.

8.2.2 List of Graphs

- Boxplots of neutralizing antibody titers by virus, visit day, and treatment arm. [*Treatment groups side by side per virus, one clade or tier and one visit per graph*.]
- Spaghetti plots of neutralizing antibody titers over time by cell type, isolate, and treatment arm.
- Magnitude-breadth (M-B) plots of titer and breadth for a panel of viruses. [*One assay type and one visit per graph.*]

8.3 B Cell Phenotyping (BCP)

The vaccine-specific B cell response is monitored using the BCP assay. The BCP assay measures expression of phenotypic markers by antigen-specific B cells from PBMC samples. To assess positivity for the detection of Trimer AB05 gp140 Env protein and Trimer 4571 B cells and IgG B cells, a Fisher's exact test will be used: A two-by-two contingency table is constructed comparing the post-vaccination and baseline (visit 1) data. The four entries in each table are (1) the number of Env-specific (IgG) B cells after vaccination (2) the number of (IgG) B cells that are not Env-specific after vaccination and (3) the number of Env-specific (IgG) B cells at baseline (4) the number of (IgG) B cells that not Env-specific at baseline. A one-sided Fisher's exact test will be applied to the table, testing whether the percent of Env-specific (IgG) B cells post-vaccination is equal to that at baseline, versus an alternative hypothesis that it is greater. Because the sample sizes (i.e., total cell counts for the B cell subset) are large, the Fisher's exact test has

high power to reject the null hypothesis for very small differences. Therefore, the p-value significance threshold is chosen stringently (≤ 0.00001). PBMC samples from visit 1 (baseline), visit 5 (2 weeks post 2nd vaccination), visit 7 (2 weeks post 3rd vaccination), visit 9 (2 weeks post 4th vaccination), and optionally visit 10 (6 months post 4th vaccination) will be tested.

The phenotypic markers to be reported are:

- % Trimer 4571+ of IgD- B cells
- % Trimer 4571+ of IgG+- B cells
- % Trimer 4571+ of IgA+ B cells
- % Plasmablasts of B cells
- % Trimer 4571+ of Plasmablasts
- % Trimer AB05+ of IgD- B cells
- % Trimer AB05+ of IgG+ B cells
- % Trimer AB05+ of IgA+ B cells
- % Trimer AB05+ of Plasmablasts

8.3.1 List of Tables

- Response rate table by B cell phenotype, visit number (visit month), and treatment group.
- Summary statistics (minimum, 25th percentile, median, 75th percentile, maximum, mean, standard deviation) among all participants, by phenotype, visit number (visit month), and treatment group.
- Summary statistics (minimum, 25th percentile, median, 75th percentile, maximum, mean, standard deviation) among positive responders by phenotype, visit number (visit month), and treatment group.

8.3.2 List of Graphs

- Boxplots (on all participants and positive responders only) of % HIV-1 Env-specific B cell by protein, visit number (visit month), and treatment group.
- Spaghetti plots of % HIV-1 Env-specific B cell by protein and treatment group.
- Reverse CDFs of % HIV-1 Env-specific B cell by protein, visit number (visit month), and treatment group.

8.4 Antibody-Dependent Cell-mediated Cytotoxicity (ADCC)

The established Luciferase and GranToxiLux (GTL) assays will be used to assess response rates and magnitude of serum ADCC. ADCC assays will be performed on participants' sera for visit 1, visit 7 (M3.5), and visit 9 (M6.5). IIf sufficient responses are seen against BG505, additional luciferase assays may be done to explore breadth of responses.

For the Luciferase assay, CEM.CCR5.NKR cells infected with HIV-1 IMC BG505 will be tested in a 96-well plate. Participant sera in addition to controls will be incubated with the IMC-infected cells, and ADCC will be detected through the use of Viviren luminescence. To assess breadth,

CEM.CCR5.NKR cells infected with HIV-1 CAP8, CH058, SUMA, and WITO, and possibly three additional IMCs, individually, will be tested.

For the GTL assay, CEM.CCR5.NKR cells coated with Clade A BG505 gp120 will be tested in a 96-well plate. Participant sera in addition to controls will be incubated with the HIV protein, and ADCC will be detected through the use of granzyme B substrate.

The following assay readouts will be used in the analyses for these assays:

i) Luciferase: The data from the luciferase assay is semiquantitative. The peak of specific killing and area under the curve (AUC) will be summarized by treatment group.

ii) GTL: The data from the assay is quantitative. The frequency of positive responses (% responders) peak ADCC, and area under the curve (AUC) will be summarized by treatment group.

Positivity criteria are as follows:

i) Luciferase: peak % loss Luciferase activity ≥10%, after background subtraction of baseline response and if responses are positive at one of the first two dilutions. For example, if the first two dilutions (1:50 and 1:200) are negative, regardless of 1:800 % loss Luciferase activity, 1:800 is negative.

ii) GTL: peak activity ≥ 8%

8.4.1 List of Tables

Luciferase:

- Response rate of peak background subtracted percent killing or loss luciferase activity by antigen, visit number (visit month), and treatment arm
- Summary statistics (i.e., min, mean, median, max) of peak background subtracted percent killing or loss luciferase activity among all participants by antigen, visit, and treatment arm
- Summary statistics (i.e., min, mean, median, max) of peak background subtracted percent killing or loss luciferase activity among positive responders by antigen, visit, and treatment arm
- Summary statistics (i.e., min, mean, median, max) of pAUC of background subtracted percent killing among all participants by treatment group, protein, and visit
- Summary statistics (i.e., min, mean, median, max) of pAUC of background subtracted percent killing among positive responders by treatment group, protein, and visit

GTL:

- Response rate of peak activity by antigen, visit number (visit month), and treatment arm
- Summary statistics (i.e., min, mean, median, max) of peak activity among all participants by antigen, visit, and treatment arm
- Summary statistics (i.e., min, mean, median, max) of peak activity among positive responders by antigen, visit, and treatment arm

- Summary statistics (i.e., min, mean, median, max) of AUC by treatment group, protein, and visit
- Summary statistics (i.e., min, mean, median, max) of AUC among positive responders by treatment group, protein, and visit

8.4.2 List of Graphs

Luciferase:

- Barcharts of ADCC Luciferase response rates and boxplots of ADCC response magnitudes by study visit, treatment group and antigen
- Barcharts of ADCC Luciferase response rates and boxplots of pAUC of background subtracted percent killing by study visit, treatment group and antigen
- When visit 9 data are available with at least 20% response rate, spaghetti plots of ADCC response magnitudes over time by treatment group and antigen.
- When visit 9 data are available with at least 20% response rate, spaghetti plots of pAUC of background subtracted percent killing over time by treatment group and antigen.

GTL

- Barcharts of GTL peak activity response rates and boxplots of peak activity response magnitudes by study visit, treatment group and antigen
- Barcharts of GTL peak activity response rates and boxplots of AUC by study visit, treatment group and antigen
- When visit 9 data are available with at least 20% response rate, spaghetti plots of peak activity response magnitudes over time by treatment group and antigen.
- When visit 9 data are available with at least 20% response rate, spaghetti plots of AUC over time by treatment group and antigen.

8.5 Infected cells antibody binding assay (ICABA)

The ICABA assay is conducted to address the exploratory objective to evaluate Ab avidity and Fc Receptor functions. ICABA will detect the presence of antibodies binding to the surface of HIV-1 infected cells in the serum of participants using Infectious Molecular Clone (IMC)-infected target cells.

The ICABA assays will be performed on participants' sera for visit 1, visit 7 (M3.5), and visit 9 (M6.5). CEM.CCR5.NKR cells infected with HIV-1 IMC BG505 and mock-infected cells will be tested in a 96-well plate. Participant sera in addition to controls will be incubated with the IMC-infected cells and the presence of bound antibodies will be detected on the surface of the cells using flow cytometry. The % of IgG+ cells is considered positive if the infected cell mock- and baseline-subtracted %IgG+ at 1:100 dilution is >5%.

8.5.1 List of Tables

- Response rate table by visit number (visit month) and treatment arm

- Summary statistics (i.e., min, mean, median, max) among all participants by visit and treatment arm
- Summary statistics (i.e., min, mean, median, max) among positive responders by visit and treatment arm

8.5.2 List of Graphs

- Barcharts of response rates, and boxplot of mock- and baseline-subtracted BG505-specific %IgG+ by visit and treatment arm.
- When visit 9 data are available with at least 20% response rate, spaghetti plot of mock- and baseline-subtracted BG505-specific %IgG+ over time by treatment arm.

8.6 Binding Antibody Multiplex Assay (BAMA)

The Binding Antibody Multiplex Assay (BAMA) assay will evaluate binding antibody responses of each serum specimen against BG505 SOSIP (vaccine trimer immunogen) and Trimer 4571 (vaccine matched immunogen). The time points to be tested are: Visit 1 (baseline), Visit 5 (Month 1.5, 2 weeks post 2nd vaccination), Visit 7 (Month 3.5, 2 weeks post 3rd vaccination), 9 (Month 6.5, 2 weeks post 4th vaccination), and Visit 10 (Month 12, 6 months post 4th vaccination). Specimens from other time points as well as other HIV antigens and antibody isotypes may be assayed based on the results of the initial assay.

The following readouts, if included in the final Central Assay Plan, will be analyzed for this assay:

i. Binding Mean Fluorescence Intensity (MFI): The frequency of positive responders (% responders) will be tabulated to assess HIV binding antibody responses between vaccine groups from MFI readouts. Positivity criteria include (1) the net MFI (MFI – Blank) 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, and (3) the MFI values are greater than 3 times the baseline MFI values.

ii. Titration (EC50/AUC): If high binding magnitude is observed and it is deemed scientifically important by the lab PI, samples may be titrated to calculate antibody titers. The geometric mean titer will be compared between vaccine groups. The overall difference in geometric mean titer between groups at each time point assays will be compared.

iii. Differential Binding Response: Epitope specificities may be assessed via wildtype-mutant pairs.

8.6.1 List of Tables

- Response rate table by isotype, antigen, visit, and treatment arm
- Summary statistics (i.e., min, mean, median, max) of net MFI among all participants (positive and negative responders) by isotype, antigen, visit, and treatment arm
- Summary statistics (i.e., min, mean, median, max) of net MFI among responders by isotype, antigen, visit, and treatment arm
- Summary statistics of binding antibody titers (AUC) among all participants by isotype, antigen, visit, and treatment arm

- Summary statistics of binding antibody titers (AUC) among positive responders by isotype, antigen, visit, and treatment arm
- Differential binding response rate table by differential binding antigen pairs, timepoint, and treatment group

8.6.2 List of Graphs

- Bar charts of response rates and boxplots of net MFI values by isotype, antigen, visit, and treatment arm
- Bar charts of response rates and boxplots of AUC by isotype, antigen, visit, and treatment arm
- Boxplots of Base AUC Magnitudes for positive differential binding responders
- Scatterplot of Base AUC vs. Trimer Env AUC
- When visit 9 data are available with at least 20% response rate, spaghetti plots of net MFI values over time by isotype, antigen, visit, and treatment arm
- When visit 9 data are available with at least 20% response rate, spaghetti plots of AUC over time by isotype, antigen, visit, and treatment arm

8.7 Electron Microscopy Polyclonal Epitope Mapping (EMPEM)

Polyclonal antibodies (IgG) are isolated from blood samples of pre-clinical or clinical HIV vaccine trial participants. The antibodies are enzymatically digested into the fragment antigen binding (Fab) components and incubated with soluble, HIV Env trimer proteins, ideally matched to the immunogen used in the study. The complex is size-exclusion chromatography purified, adsorbed onto electron microscopy (EM) grids, and imaged. Individual protein complex particles are extracted from the images and subjected to averaging and classification in 2D and 3D space.

The resulting 3D EM maps are matched to known structures of Env in complex with antibodies and each polyclonal Fab specificity is assigned an epitope label based on overlap with known structure(s). The epitopes detected are: gp41-base, V1V2V3, gp41-GH, C3V5, CD4bs, gp120-GH, gp120-gp120, gp41-FP, and protomer. The results consist of the following: a) binary indicators of epitope-specific antibodies detected; b) antibody-induced trimer disassembly is detected if protomers are visible during 2D classification; c) a relative abundance value of each epitope-specific antibody observed in trimer-Fab complexes. Summaries of response are number of epitope-specific antibodies detected, the relative abundance of each epitope-specific antibody in a given sample, and number of epitope-specific antibodies excluding responses detected to the gp41-base. Serum specimens are collected from enrolled participants at Visit 5 (Month 1.5, 2 weeks post 2nd vaccination), Visit 7 (Month 3.5, 2 weeks post 3rd vaccination), and Visit 9 (Month 6.5, 2 weeks post 4th vaccination).

8.7.1 List of Tables

- Response rate table by epitope, visit number (visit month), and treatment group.
- Response rate table of antibody-induced trimer disassembly (detected protomers) by visit number (visit month) and treatment group.
- Summary statistics (mean, median) of epitope-specific antibodies (with and without responses directed to the base, excluding protomers) by treatment group and visit number (visit month)

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8.7.2 List of Graphs

- Bar graphs of response rate and boxplots of magnitude of response by epitope, visit number (visit month) and treatment group
- Bar graph of antibody-induced trimer disassembly (detected protomers) by visit number (visit month) and treatment group
- Individual-level data plot of epitopes detected with participants as rows and epitopes as columns and including protomer responses (beginning with base and subsequent epitopes displayed in alphabetical order), faceted by treatment group and visit number (visit month)
- Boxplots of the number of epitope-specific antibodies (with and without responses directed to the base, excluding protomers) by treatment group and visit number (visit month)

9 REFERENCES

1. Agresti A, Coull BA. Approximate is better than "exact" for interval estimation of binomial proportions. Am Stat. 1998;52(2):119-26.
2. Hughes JP. Mixed effects models with censored data with application to HIV RNA levels. Biometrics. 1999;55(2):625-9.
3. Rotnitzky A, Robins J. Analysis of semi-parametric regression models with non-ignorable non-response. Stat Med. 1997;16(1-3):81-102.

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