

# Laboratory

## 1.1 Endpoint assays: cellular

### 1.1.1 Flow cytometry

Flow cytometry will be used to examine vaccine-specific CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses following stimulation of PBMCs with synthetic HIV peptides that span the proteins encoded by the vaccine. Data will be reported as percentages of CD4<sup>+</sup> or CD8<sup>+</sup> T cells responding to a specific peptide pool. Additional cell surface markers, cytokines, or functional markers may also be analyzed.

### 1.1.2 IFN- $\gamma$ ELISpot

PBMCs will be stimulated overnight with synthetic peptide pools that span the proteins included in or encoded by the vaccine. This process will allow ex vivo HIV-specific T-cell data to be assessed by IFN- $\gamma$  ELISpot as an immunogenicity endpoint. Data will be reported as the number of spot-forming cells (SFC) per 10<sup>6</sup> cells recognizing a specific peptide pool.

### 1.1.3 CD4<sup>+</sup> T follicular helper cells (Tfh/pTfh)

Multicolor flow cytometry will be used to identify and phenotype circulating CD4<sup>+</sup> T cells in the peripheral blood (PBMC) and LN FNAs (if applicable). Functionality of Tfh cells might be tested by in vitro stimulation of cells with synthetic HIV peptides that span the HIV-1 envelope protein to identify, enumerate and immunophenotype antigen-specific Tfh cells.

### 1.1.4 Antigen-specific B cell and plasmablast phenotyping

HIV-1 antigen-specific memory B cells and plasmablasts induced by vaccination will be identified and characterized using fluorescently-labeled recombinant proteins in combination with a flow cytometry phenotyping panel. In particular, HIV Env-reactive B cells and plasmablasts will be enumerated and may be further characterized for expression of memory, activation, inhibitory or other markers of interest.

### 1.1.5 B-cell receptor sequencing

Single or bulk populations of memory B cells and plasmablasts may be sorted for BCR sequencing and gene expression analysis. Env-specific B cells may be expanded for detection of and functional testing of secreted antibodies by enzyme-linked immunosorbent assay (ELISA) or microneutralization assays, and BCR sequencing. The resulting VH and VL genes may be cloned into an IgG backbone for antibody expression and characterization of the binding, epitope-specificity, and/or neutralization.

### **1.1.6 Epitope mapping**

Epitope mapping will be performed using either the ICS or IFN- $\gamma$  ELISpot assays. PBMCs will be stimulated with synthetic peptides that span the proteins included in or encoded by the vaccine. Once positive responders are identified using peptide pools, the specific responses may be mapped to single peptides.

## **1.2 Endpoint assays: humoral**

### **1.2.1 Binding antibody multiplex assay (BAMA)**

HIV-1-specific total binding IgG antibodies will be assessed on serum samples from study participants. In addition, HIV-1-specific total binding IgA antibodies and binding to IgG subclasses (IgG1, IgG2, IgG3, and IgG4) may also be assessed.

### **1.2.2 Antibody avidity**

Antibody avidity may be measured using BAMA with the addition of a dissociation step to calculate the antibody avidity index (BAMA-AI). Biolayer Interferometry (BLI) and/or Surface Plasmon Resonance (SPR) technologies may also be used to define antibody avidity.

### **1.2.3 Antibody-dependent cellular cytotoxicity assay (ADCC)**

ADCC activity may be assessed using serum samples from study participants. For the Granzyme B flow-based cytotoxicity assay, participant sera are incubated with effector cells sourced from human PBMCs and gp120-coated CEM.NKR.CCR5 target cells. ADCC is quantified as net percent granzyme B activity which is the percent of target cells positive for GranToxiLux (GTL), an indicator of granzyme B uptake, minus the percent of target cells positive for GTL when incubated with effector cells but without sera. For the Luciferase-based cytotoxicity assay, participant sera are incubated with infectious molecular clone (IMC)-infected CEM.NKR.CCR5 cells and percent killing is measured through the use of Viviren luminescence.

### **1.2.4 Antibody-dependent cellular phagocytosis (ADCP)**

To assess the ability of vaccine-elicited antibodies to engage cellular FcR for potential antiviral function, ADCP may be measured using serum samples from study participants. ADCP is measured by assessing the ability of vaccine elicited antibodies to mediate monocyte phagocytosis of HIV-1 antigen coated fluorescent beads by flow cytometry (26, 27). An array of antigens may also be analyzed at the discretion of the HVTN Laboratory Center.

### **1.2.5 Antibody-dependent neutrophil phagocytosis (ADNP)**

To assess the ability of vaccine-elicited antibodies to engage cellular FcR for potential antiviral function, ADNP may be measured using serum samples from study participants. ADNP is measured by assessing the ability of vaccine elicited

antibodies to mediate neutrophil cell line phagocytosis of HIV-1 antigen coated fluorescent beads by flow cytometry (28). An array of antigens or viruses may also be analyzed at the discretion of the HVTN Laboratory Center.

### **1.2.6 Complement assays**

The capacity of antibodies to bind complement will be assessed either through a direct binding assay or a complement deposition assay (29, 30). The complement deposition assay is a cell-based method. The binding assay is a bead-based multiplexed method that measures engagement of antigen specific antibody with complement.

### **1.2.7 Infected cells antibody binding assay (ICABA)**

The capacity of vaccine elicited antibodies to recognize epitopes exposed on the surface of infected cells may be assessed using serum samples from study participants. This assay measures the capacity of vaccine elicited antibodies to recognize HIV envelope on the surface of infected cells (ie, the first step in mediating antibody Fc effector function) with a readout by flow cytometry.

### **1.2.8 Fc receptor binding**

The ability of vaccine elicited antibodies to bind to cellular FcRs enables characterization of the antibody Fc profile that results in antiviral function (ie, includes subclass and glycans). HIV-specific antibody binding to Fc $\gamma$ R proteins will be assessed by the FcR BAMA. The FcR BAMA is a modification of the binding assay where fluorescently labeled FcR proteins are utilized as the detection reagent for serum antibodies bound to HIV proteins on microspheres. The readout may include the magnitude and/or avidity for an array of FcRs involved in mediating antiviral activity.

### **1.2.9 Peptide microarray**

Linear epitope specificities of purified serum IgG will be examined by peptide microarray using an Env peptide library, which contains 15-mer peptides that overlap by 12 amino acids and cover consensus Env strains and vaccine strains.

### **1.2.10 Neutralizing antibody assay**

HIV-1-specific nAb assays will be performed on serum samples from study participants. The TZM-bl assay will test neutralization of the vaccine strain(s) [insert strain(s)] and a single highly neutralization-sensitive Tier 1 virus as a positive control (insert strain). The global panel and/or clade-specific panels may be used to assess Tier 2 neutralization. (21, 31)

### **1.3 Innate immunity assays**

#### **1.3.1 Soluble factors in serum or plasma**

Multiplex cytokine assays and/or enzyme-linked immunosorbent assay (ELISA) may be used to measure soluble cytokines, chemokines, and other immunomodulatory factors in the serum or plasma. Analytes may include IFN- $\gamma$ , IL-6, TNF-  $\alpha$ , IL-10, IFN- $\gamma$  inducible protein 10 (IP-10), and/or monocyte chemoattractant protein 1 (MCP-1). Other analytes may also be included.

#### **1.3.2 Enumeration and phenotyping of cell populations**

Phenotyping of DCs, monocytes, NK cells, B cells, T cells, or other leukocytes for lineage, maturation, and activation markers may be performed on fresh or cryopreserved PBMC. Trucount tubes with whole blood will be used when possible for direct enumeration of major leukocyte populations, including DC, by flow cytometry. Data will be reported as cell concentrations per microliter of blood and/or as percentage of live cells.

#### **1.3.3 Gene expression**

Bulk PBMC or whole blood will be cryopreserved in an RNA protection reagent. RNA may be isolated and used for analysis by RNA sequencing and/or real-time PCR. Signatures of gene expression changes will be analyzed over time after vaccination.

### **1.4 Genotyping**

Molecular human leukocyte antigen (HLA) typing may be performed using PBMC samples. Other genes, including those associated with immune responses (eg, immunoglobulin or T cell receptor genes) or HIV-1 disease progression may also be assessed.

### **1.5 HVTN Lab Center assay portfolio**

Additional assays may be performed per the HVTN Laboratory Center assay portfolio, which includes immune assessments such as those for cellular, humoral, and innate immune responses, and host genetics. The assay portfolio will be updated periodically to include new assays and adjust qualification levels of existing assays.