REVIEW ARTICLE

Current Approaches for Assessments of Neutralizing, Binding, and Effector Functions of Antibodies on the Path to Antibody-Mediated Prevention Strategies for HIV-1

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Abstract: Robust assay technologies and reference reagents are essential components in efforts to develop safe and effective antibody-mediated prevention strategies for HIV-1. Here, we describe current approaches used to conduct standardized assessments of neutralizing, binding, and Fc receptor-mediated effector functions of vaccine-elicited antibodies, with an emphasis on recent developments that enable early precursors and intermediates of broadly neutralizing antibodies (bnAbs) to be monitored. We also describe how these assay technologies were adapted to facilitate clinical evaluations of passively delivered bnAbs for HIV-1 prevention.

Keywords: Neutralizing, binding effector, antibodies, antibody-mediated prevention, HIV-1, vaccine-elicited antibodies.

1. INTRODUCTION

Efforts to develop a safe and effective HIV-1 vaccine place a heavy emphasis on an ability to elicit durable, high titers of broadly neutralizing antibodies (bnAbs) as the most effective means to prevent HIV-1 acquisition and put an end to the pandemic. The first 20 years of HIV-1 vaccine development saw little progress toward this goal, owing in large part to a paucity of information on relevant epitopes and antibody lineages to target and because of a reliance on monomeric gp120 immunogens that failed to engage the correct naïve B cell receptors to initiate bnAb development. Considerable advances over the last 15 years have provided a wealth of information to guide the design of viable immunogens [1, 2]. Thus, native-like envelope glycoprotein (Env) trimers stabilized in a prefusion conformation (*e.g.*, SOSIP design) have succeeded at generating autologous tier 2 virus-neutralizing antibodies where previous monomeric gp120 immunogens failed to do so [3-5]. In a more recent proof-of-concept study, a germline-targeting immunogen (eOD-GT8 60mer) elicited early precursors of CD4 binding site (CD4bs) bnAb lineages in humans [6]. Scientific principles leading to these successes provide a foundation to elicit early precursors of other epitope classes of bnAbs, with promising results obtained in human gene knock-in mouse models and nonhuman primates [7-15]. As a result, the HIV-1 Vaccine Clinical Trials Network (HVTN) has nearly 20 active and planned proof-of-concept discovery medicine trials testing immunogens targeting bnAb epitopes in the CD4bs, V2-apex and V3-glycan of gp120 and the fusion peptide and membrane proximal external region (MPER) of gp41 (www.hvtn.org). A subset of immunogens in these trials is given the term "germline targeting" because they are specifically designed to activate B cells bearing germline versions of the correct antibody lineages known to give rise to bnAbs. Once activated, subsequent boosting immunogens aim to drive the requisite somatic mutation and affinity maturation for full bnAb activity [16-18]. Progress toward bnAb development is monitored serologically using a variety of sophisticated techniques described in this article, which complement phenotypic and genetic interrogations at the B cell level.

In addition to their importance for vaccines, bnAbs as monoclonal antibodies delivered passively are of interest as an option for pre-exposure prophylaxis (PrEP) of HIV-1 infection. In an important proof of concept study, repeated intravenous administrations of the CD4bs bnAb, VRC01, was safe, well-tolerated, and demonstrated 75% prevention efficacy against highly VRC01-sensitive HIV-1 (VRC01 IC80 <1 µg/ml) in the Antibody Mediated Protection (AMP) trials [19]. This demonstration of neutralizing antibody-mediated protection against the acquisition of HIV-1 in humans lends legitimacy to the TZM-bl assay as a correlate of protection and yielded a serum neutralization titer biomarker for use in predicting HIV-1 prevention efficacy [20]. Thus, prevention of infection against an exposing strain of HIV-1 is predicted

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to require a serum neutralization ID80 titer in the TZM-bl assay of 200 (PT80= 200). PT80 provides a benchmark for neutralizing antibody-mediated prevention of HIV-1 infection in the setting of both active and passive immunization.

Encouraged by the AMP results, efforts are underway to test a complementary combination of bnAbs predicted to exceed the prevention efficacy of VRC01 [21]. An early study of 22 combinations of four epitope classes of bnAbs assayed against a multi-clade panel of 125 viruses found that the greatest magnitude and breadth of neutralization could be achieved with three bnAbs, each to a different epitope [22]. Subsequent studies with larger datasets covering a wider spectrum of bnAbs identified optimal combinations of best-in-class bnAbs of greatest interest for clinical development [23, 24]. The current emphasis for pending efficacy trials combines three bnAbs targeting the CD4bs (VRC07.523LS), V2-apex (PGDM1400LS), and V3-glycan (PGT121.414LS) epitopes [20, 21, 25]. BnAbs for future "combo AMP" efficacy trials are engineered for extended serum half-lives by introducing methionine to leucine (L) and asparagine to serine (S) changes (LS mutation) in their Fc region [26, 27].

BnAbs and certain non-neutralizing antibodies have also the potential to mediate antiviral effector functions through the engagement of Fc receptors; such effector functions mediated by gp120 V1V2-specific non-neutralizing antibodies correlated with the apparent modest efficacy of a bivalent gp120 vaccine in the RV144 trial [28]. Because follow-up vaccine trials lacked efficacy [29-31], interest in non-neutralizing antibody-based vaccines has waned in recent years. Nonetheless, analyses of immune correlates of risk in these follow-up efficacy trials continued to show V1V2 antibodies corresponding to a decreased risk of HIV-1 infection [32, 33]. The level of antiviral activity by these antibodies may only provide a modest level of efficacy when in isolation; however, induction of antibody Fc effector functions could boost the overall level of vaccine efficacy of a first-generation bnAb-inducing vaccine regimen. A first-generation bnAb-inducing vaccine regimen may only generate moderate neutralization breadth and potency against circulating viruses in the majority of people. Strategies to evaluate multiple antiviral activities of HIV-1 specific antibodies, including neutralizing and antibody Fc effector functions, will enable progress toward highly efficacious vaccine regimens.

Here we describe how neutralizing, binding, and effector functions of antibodies are assessed in a laboratory program at Duke University that adheres to Good Clinical Laboratory Practices (GCLP) and has a long history of supporting the HVTN, the Gates Foundation Collaboration for AIDS Vaccine Discovery (CAVD), the preclinical HIV-1 vaccine portfolio of DAIDS-NIAID-NIH, and other HIV-1 vaccine programs. We emphasize both vaccine-elicited and passively delivered antibodies for HIV-1 prevention.

1.1. TZM-bl Assay for Neutralizing Antibodies

Reporter gene assays with Env-pseudotyped viruses have been the gold standard for HIV-1 neutralizing antibodies for nearly two decades. The most widely used assay of this kind measures neutralization as a function of reductions in Tat-regulated luciferase reporter gene expression after a single round of infection in TZM-bl cells [34] (Fig. **1**). Although the TZM-bl assay can utilize uncloned replication-competent primary isolates, clonal Env-pseudotyped viruses are preferred because they allow detailed analyses and experimental manipulation (*e.g.*, site-directed mutagenesis) at the genetic level. TZM-bl cells are derived from HeLa cell clone HI-J, which naturally expresses CXCR4 and was engineered to express high levels of CD4 [35] and CCR5 [36]. A clone of HeLa-CD4-CCR5 (clone JC53) was further engineered to contain reporter genes for firefly luciferase and *E. coli* β-galactosidase under control of the HIV-1 LTR [37]. This reporter cell line was originally called JC53BL-13 and later became known as TZM-bl. The utility of the TZM-bl assay for studies of HIV-1 neutralizing antibodies was first demonstrated in a study of autologous neutralization and escape in people living with HIV-1 [38]. A related study using a similar assay technology but with a different cell line was reported at about the same time [39]. Both assays generated nearly equivalent results when applied to one of the earliest HIV-1 vaccine efficacy trials [40]. The TZM-bl assay was optimized, qualified, and validated at Duke University [41] and transferred to over 50 laboratories around the world [42]. An international TZM-bl assay proficiency testing program was implemented in 2009, jointly sponsored by the Gates Foundation and DAIDS-NIAID-NIH, in which participating laboratories are formally tested twice annually as a requirement for GCLP compliance [43].

Results in the TZM-bl assay generally agree with those obtained in other assays but tend to be more sensitive and reproducible [44, 45]. An ad hoc committee comprised of approximately 50 scientists met in 2004 and overwhelmingly embraced a transition to pseudovirus/reporter gene assays and made recommendations for the design of suitable panels of reference strains [46], which were in short supply at the time.

1.2. HIV-1 Env-pseudotyped Reference Strains

The extraordinary genetic and antigenic variability of HIV-1 are major obstacles for vaccines and also pose challenges for reference strain selection. Moreover, the trimeric Env spike of HIV-1 is structurally dynamic, spontaneously transitioning between "open" and "closed" conformations at different frequencies that differentially effect the neutralization phenotype of the virus [47]. Most circulating strains of HIV-1 exhibit a neutralization phenotype categorized as tier 2 that is associated with a predominantly closed trimer structure [48, 49]. Rare isolates possess a tier 1 phenotype with a more open trimer that is susceptible to neutralization by many antibody specificities that do not neutralize tier 2 viruses and are unlikely to protect against HIV-1 acquisition [40]. Tier phenotyping is, therefore, an important requirement for reference strain selection and is typically accomplished by using a panel of HIV-1-positive serum samples with predefined ranges of potency that differentiate the tier phenotypes of HIV-1. Tier 2 viruses, being the most relevant targets for vaccines, are the most important reference strains for immune monitoring.

An initial panel of twelve Env-pseudotyped viruses was quickly developed as reference reagents to facilitate standardized datasets [50]; these viruses were from acute and early clade B HIV-1 infections and exhibited a tier 2 neutralization phenotype, but it was not known how well they represented

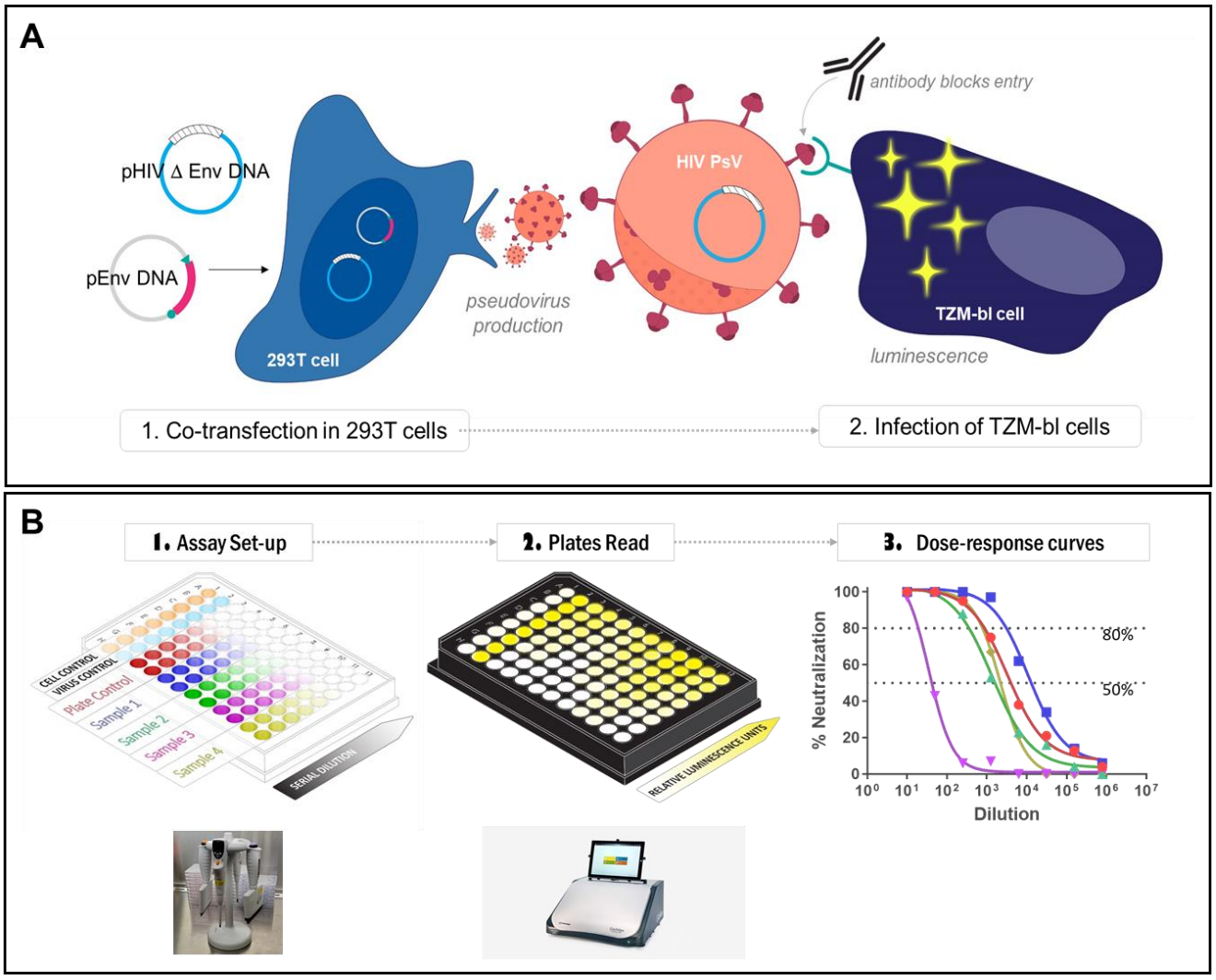

Fig. (1). TZM-bl neutralizing antibody assay for HIV, SIV and SHIV. (**A**) Env-pseudotyped viruses are produced in 293T cells by co-transfection with a plasmid expressing the Env of choice and a second plasmid encoding the entire HIV-1 genome with a defective Env gene. Pseudovirions package the backbone plasmid for expression of Tat and induction of a luciferase reporter gene once inside TZM-bl cells. Reductions in luciferase activity, measured as relative luminescence units (RLUs) after addition of substrate, are directly proportional to the degree of virus neutralization. (**B**) The assay is performed in 96-well culture plates for high throughput. Samples are serially diluted (*e.g.*, 3-fold or 5-fold) in duplicate for a total of 8 dilutions, followed by the addition of a pre-titrated dose of virus and a brief incubation period before adding TZM-bl cells. One set of wells receives cells only (background), while another set of wells receives cells and virus only (maximum infection). RLUs are measured after 2 days of incubation and used to construct neutralization curves for determination of 50% and 80% inhibitory dilutions (ID_{50} and ID_{80}, respectively). Diagram by Francesca Suman. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

the spectrum of circulating viruses, even within the B clade. Later, with generous support from multiple partners, hundreds of high-fidelity gp160 clones from tier 2 viruses representing all major genetic subtypes and circulating recombinant forms of HIV-1 from high-incidence geographic regions were widely available for use as Env-pseudotype viruses [49, 51-54]. Computational analysis of a large neutralization dataset generated with a multi-clade panel of 219 Env-pseudotyped viruses and 205 HIV-1 serum samples identified a subset of 9 viruses that most accurately predict the spectrum of activity in the larger dataset [51]. This small "global" reference panel was widely distributed and routinely used to evaluate the magnitude and breadth of tier 2 virus neutralization in preclinical and clinical HIV-1 vaccine studies. A similar analysis of 200 clade C Env-pseudotyped viruses from southern Africa identified a complementary panel of 12 reference strains for greater representation of the geographic region most affected by HIV-1 [49]. Both down-selected panels of reference strains enable reliable comparisons across datasets. Such comparisons benefit from robust statistical methods designed for the analysis of neutralization magnitude/breadth curves [55]. The inclusion of greater numbers of reference viruses enables large-scale assessments of neutralization magnitude and breadth that help identify lead candidate bnAbs for clinical development [22-24]. Site-directed mutations known to knockout bnAb activity were introduced into the two smaller panels of viruses to map the epitope specificity of tier 2 virus neutralization.

As HIV-1 continues to diversify, reference strains from early in the pandemic may need updating to account for a slow but steady trend toward decreasing sensitivity to bnAbs over time [49, 56]. Most of the 219 multi-clade Envs mentioned above were isolated between 1992 and 2007, while the 200 clade C Envs were isolated between 1998 and 2010. Two recent studies examined antigenic drift of bnAb epitopes using Envs from placebo recipients in the AMP trials who acquired HIV-1 between 2017 and 2019. AMP placebo viruses from southern Africa (subtype C) were less sensitive to VRC07-523LS (CD4bs) and CAP256.25 (V2-apex) compared to older clade C viruses [57]. Likewise, AMP placebo viruses from the Americas (subtype B) were less sensitive to several CD4 binding site bnAbs and bnAbs targeting V3-glycan, V2-apex, and fusion peptide epitopes compared to older clade B viruses (K. Wagh *et al.*, in preparation). These recent findings are consistent with earlier findings [49, 56] and point to the benefits of updated reference strains for neutralization assays.

The AMP placebo viruses were an opportunity to create contemporary panels of reference strains, albeit limited to clades B and C. A total of 55 clade B Envs from placebo recipients in HVTN 704/HPTN 085 (AMP in the Americas) and 46 clade C Envs from placebo recipients in HVTN 703/HPTN 081 (AMP in southern Africa), all transmission/founder lineage viruses, were assayed with 15 bnAbs targeting CD4bs, V3-glycan, V2-apex, fusion peptide and MPER bnAb epitopes. A subset of 12 viruses was computationally selected as updated reference strains (REP-T2: The REPresentative screening panel, Tier 2) to represent the spectrum of diversity of bnAb sensitivity/resistance across all of the AMP placebo viruses. A separate subset of 12 viruses was computationally selected (SHEP-T2: The Sensitive HIV-1 Envelope Panel, Tier 2) that includes the top two ranked viruses that are most sensitive to each bnAb class. The purpose of this latter panel is to maximize the ability to detect low levels of heterologous tier 2 virus neutralization regardless of the bnAb classes induced, keeping in mind the viruses are not intended to closely approximate the natural diversity of contemporary viruses in the way the REP-T2 panel is designed to do. Because some vaccine immunogens target specific epitope classes of bnAbs, additional panels of reference strains were selected, each designed for maximum detection of a different epitope class of bnAbs.

1.3. Monitoring Early Precursors of bnAbs using the TZM-bl Assay

With the growing number of germline-targeting immunogens entering the preclinical and clinical pipelines has come a need to develop specialized laboratory methods to monitor progress. Early precursors of most bnAbs do not acquire neutralizing activity until they reach later stages of maturation, rendering the TZM-bl assay ineffective until substantial progress leads to detectable neutralization against tier 2 reference strains. In order to employ the assay sooner and facilitate neutralization-guided progress, engineered Env-pseudotyped were created that detect neutralization signatures indicative of the presence of early precursors and intermediates of bnAbs. Pseudoviruses were engineered to have some of the same features used to create germline-targeting immunogens, including targeted glycan deletion and glycan truncation to allow easier access of precursor antibodies to their epitopes, and non-glycan mutations involving known key residues of bnAb epitopes. While this is a work in progress, we describe below our progress to date using engineered pseudoviruses and corresponding epitope knockout versions to detect neutralization signatures of early precursors and intermediates of a subset of bnAbs of interest. The results inform the potential for the success of germline targeting vaccine strategies and identify interesting vaccine recipients for deep interrogation of the B cell response that gave rise to the signature activity.

1.3.1. VRC01 Precursor Detection

VRC01 is a VH1-2 restricted (CD4-mimicking) CD4bs bnAb [58, 59]. Near germline versions of VRC01-class bnAbs neutralize the 426c strain of HIV-1 in a manner that requires the deletion of one or more N-glycans in the vicinity of the CD4bs, combined with Man_5 enrichment of N-linked glycans that are otherwise processed into large complex-type glycans [60]. Man_5-enrichment is achieved by producing the pseudovirions in cells deficient in the enzyme N-acetylglucosaminyltransferase (GnT1- cells). 293T cells are used to produce pseudoviruses with fully processed glycans. Man_5-enriched forms of 426c lacking a single glycan (426c.N276D/GnT1-) or lacking three glycans (426c.N276D.N460D.N463D/GnT1-), both produced in GnT1- cells, detect early precursors of VRC01 class bnAbs [60]. When detected, the VRC01 resistance mutation D279K is used to confirm CD4bs specificity and complete the signature. Intermediate stages of maturation are monitored by using 426c in which the glycan modifications are partially or completely reverted to wild type (*e.g.*, glycan-deleted 426c without Man_5-enrichement, Man_5-enriched 426c without glycan deletions, parental 426c produced in 293T cells). Neutralizing activity detected against either 426c.N460D.N463D/GnT1- or 426c/GnT1 indicates an ability to tolerate the N276 glycan, which is a key requirement in VRC01 class bnAb development [61, 62]. We also monitor N276 glycan toleration in the context of heterologous tier 2 viruses based on differential neutralization of virus pairs in which the glycan is either present or knocked out by site-directed mutagenesis.

Immunogens derived from 426c Env with high affinity for germline-reverted VRC01 [63, 64] are in development as germline-targeting vaccines for VRC01 class bnAbs. Other germline-targeting immunogens for VRC01 class bnAbs include eOD-GT8 [5] and BG505 SOSIP.GT1.1 [65]. Using the viruses described above, neutralization signatures of early precursors of VRC01 were detected and subsequently verified with isolated monoclonal antibodies in clinical studies of all three immunogens (unpublished). In addition, a neutralization signature of VRC01-like early precursors was detected in a recent nonhuman primate study of BG505 SOSIP.GT1.1 [66].

1.3.2. CH235.12 Lineage Detection

CH235.12 is CD4bs bnAb that utilizes VH1-46 and neutralizes 90% of viruses in a multi-clade panel of tier 2 viruses [67]. A study of virus/antibody co-evolution in donor CH505 identified a natural Env mutation, N279K (also called M5), that is thought to have participated in the early development of the CH235 lineage [67]. A gp120 V5 loop mutation, G458Y, and Man_5-enrichement (by producing virus in GnT1- cells) work synergistically with each other and with the

N279K mutation in creating an engineered variant (CH505.N279K.G458Y/GnT1-) derived from the CH505 transmitted-founder lineage virus that is highly susceptible to neutralization by germline-reverted CH235.12, with IC50 <0.1 μg/mL [68]. Neutralization by the germline-reverted antibody and early intermediates, but not mature CH235.12, is knocked-out by N280D to confirm epitope specificity [69]. When this complete neutralization signature is detected in vaccine studies, intermediate stages of maturation are monitored by using viruses in which the germline-targeting features in the CH505.N279K.G458Y/GnT1-virus are partially or completely reverted to wild-type [68, 69]. Further maturation is monitored with a panel of heterologous wild-type viruses that differentiate early, intermediate, and late-stage members of the CH235.12 lineage.

CH505.N279K.G458Y/GnT1- produced as a stabilized Env trimer, is currently under investigation as a germline-targeting vaccine strategy for CH235.12 lineage bnAbs [12]. Using the precursor detection virus, CH505.N279K.G457Y /GnT1-, and corresponding N280D knock-out mutation, a neutralization signature of early precursors of CH235.12 was detected in a nonhuman primate study of the germline-targeting immunogen [69]. Monoclonal antibodies isolated from immunized animals recapitulated the signature and showed characteristics of CH235.12 lineage usage of a rhesus orthologue of VH1-46, confirming the precursor signature found in sera. The first clinical study of this immunogen (HVTN 312) began in October 2024 and uses mRNA delivery of stabilized CH505.N279K.G458Y trimer containing an additional mutation, N197D, that removes a key glycan and thereby circumvents the need for Man5 enrichment.

1.3.3. CH103 Precursor Detection

CH103 was isolated from the same donor as CH235 (donor CH505) and is a CD4bs bnAb that utilizes VH4-59 and neutralizes 55% of viruses in a multi-clade panel of tier 2 viruses [70]. Germline-reverted CH103 do not neutralize the autologous CH505 transmitted-founder lineage virus unless four N-linked glycans at positions 197, 276, 362, and 462 in gp120 are removed and the virus is produced in GnT1-cells [68]. When activity against the CH505.gly4/GnT1-virus is detected, an epitope knockout mutation, S365P, is used to confirm CH103-like precursor activity [68].

1.3.4. DH270 Lineage Detection

DH270 is a V3-glycan bnAb isolated from donor CH848 in whom the coevolution of HIV-1 Env and the DH270 lineage was studied over time from acute infection to mature bnAb development [71]. This information provides insights for vaccine strategies soon to be tested in discovery medicine trials [12]. Removal of two glycans in the V1 region of gp120 (N133D and N138T) from virus CH848_10.17 isolated 949 days after the estimated time of infection rendered the virus (CH848_10.17DT) susceptible to neutralization by germline-reverted DH270 [12]. Parental CH848_10.17 is not neutralized until the germline-reverted antibody acquires a G57R improbably mutation [71], making it useful to monitor progress. A panel of heterologous tier 2 viruses permits additional stages of maturation to be monitored [71].

1.3.5. Vaccine-Elicited Binding Antibodies

Standardized and validated binding antibody methods rigorously compare across candidate immunogens and serve to bridge preclinical and human clinical trials for iterative HIV-1 vaccine design focused on stimulating bnAb precursors. Sensitive and specific binding antibody multiplex assays (BAMA) [28, 72, 73] detect polyclonal serum antibodies to candidate bnAb vaccine immunogens, employing well-characterized minimal immunogen, trimer (*e.g.*, SOSIP) and modified antigens to evaluate elicitation of bnAb precursors to mature bnAbs specific for the target epitope (CD4bs, V2-apex, V3-glycan, gp120-gp41 interface) *versus* off-target specificities (*e.g.*, base of trimer) (Fig. **2**). Antigens are covalently bound to microspheres *via* direct conjugation or captured via Avi-tag (*e.g.* biotinylated Avi-tagged proteins) and characterized to verify proper trimer conformation/antigenicity using a panel of germline and mature bnAbs and non-bnAbs directed to vulnerable regions on the HIV-1 envelope (*i.e.* CD4bs, CD4i, V2 glycan, V3 glycan, gp120-gp41 interface, MPER) or trimer base. Epitope-specific responses are resolved by differential binding to wild-type and epitope knockout (KO) mutant Env proteins diagnostic of known bnAb versus non-bnAb epitopes. Epitope-specific bnAbs, such as MPER-directed antibodies [74, 75], are measured with a panel of recombinant MPER peptides and gp41 proteins for epitope mapping. Core epitopes of MPER bnAbs 2F5, 4E10, and 10E8 are defined through alanine scanning mutants spanning the MPER with readouts of microgram per milliliter (μg/mL) equivalent antibody concentrations. These qualified and validated methods in a GCLP-compliant laboratory environment continue to inform the magnitude, epitope specificity, and kinetics of circulating vaccine-elicited antibodies as part of the evaluation of recent bnAb-inducing vaccine regimens [5, 6].

Antibody subclass and isotype measurements are important for understanding optimal responses that track with the engagement of FcR and that can potentially help predict bnAb maturation [76-78]. Evidence that Env IgG3 correlates with decreased risk of HIV-1 infection [33] and that Env IgA in serum negatively modulates antibody Fc effector function [33, 79, 80] support a role for continued evaluation of isotype/subclass specific responses in vaccination. For bnAb-inducing vaccine regimens, antibody class switching may be an immune mechanism enabling continued neutralization potency against diverse HIV-1 strains [78].

Binding antibody breadth is assessed via panels of Env antigens, including stabilized SOSIP trimers, representative of global HIV-1 diversity [73]. Current strategies employ transmitted/founder lineage Env sequences from the placebo arms of the AMP (HVTN 703 (clade C viruses)/HVTN 704 (clade B viruses) [19, 57], Imbokodo (HVTN 705), and Mosaico (HVTN 706) HIV-1 vaccine efficacy trials. Selections are based on genetic signatures/phenotype (*versus* historical viruses) and selecting from clusters of viruses with shared binding profiles. Trimers containing mutations in specific epitopes are used for fine mapping of distinct bnAb specificities.

Tracking the avidity maturation of circulating antibody responses after HIV-1 immunogen boosting is important for evaluating the effectiveness of stimulating specific bnAb

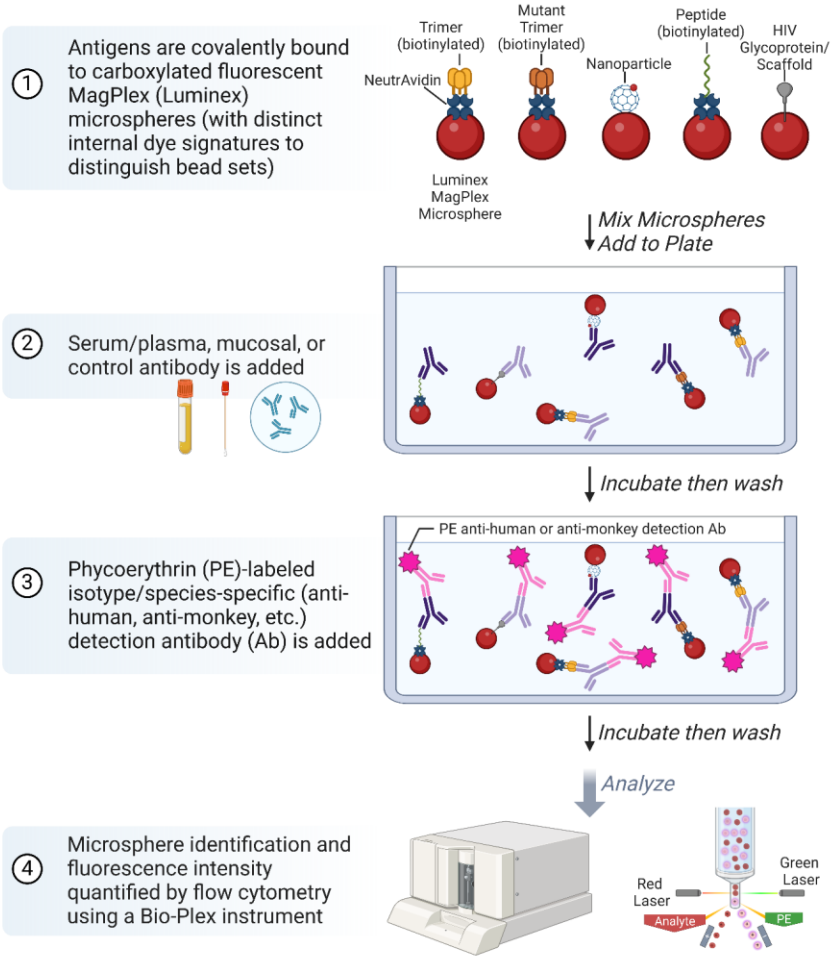

Fig. (2). Binding antibody multiplex assay (BAMA). The validated BAMA employs a custom Luminex® platform for multiplexing multiple antigens (*e.g.* up to 500 different proteins/epitopes) in a single assay run, enabling profiling of binding antibody breadth, isotype (IgG, IgA (dimeric, secretory), IgM, IgE), and subclass (IgG1-4, IgA1-2) in multiple sample types. Antigens to map HIV-1 early precursor and mature broadly neutralizing antibody (bnAb) epitopes, such as wild-type and epitope-specific mutant native-like Envelope glycoprotein trimers (*e.g.* SOSIP), scaffolds, peptides, germline targeting and/or nanoparticle-based antigens (*e.g.* eOD-GT8, 426c), are captured onto fluorescent microspheres *via* Avi-tag to NeutrAvidin (if biotinylated) or direct conjugation. Diluted samples are incubated with the microsphere mixture, followed by addition of a secondary PE-labeled isotype-specific detection antibody and reading on a Bio-Plex (Bio-Rad) instrument. The dual lasers in the instrument to identify each microsphere (red laser) and the amount of reporter dye (PE) (green laser) bound to its surface, with antibody binding reported as median fluorescence intensity (MFI) to assess specificity and magnitude. BAMA outputs include vaccine response rate, monoclonal antibody (mAb) equivalent concentration, epitope specificity (differential binding), specific activity (mucosal), Fc receptor (FcR) binding (with use of FcR detection reagents), and avidity index (with use of dissociation buffer). Limit of detection and lower limit of quantitation are specific for antibody/antigen pairs, ranging from 0.10 to 10 ng/mL and 0.2 to 20 ng/mL, respectively. Figure Created in BioRender.com. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

lineages toward broadly neutralizing activity. The avidity of polyclonal antibodies is measured by evaluating the dissociation rate constant (*kd*) of diluted serum or purified serum IgG binding to Env antigens, including minimal immunogens such as MPER or the fusion domain peptides, gp120 monomer, and gp140 trimers (*e.g.*, SOSIP trimers or other stabilized native-like constructs) [81]. The variations in polyclonal off-rates between boosting and among different immunization strategies provide readouts on affinity maturation induced by vaccination [82].

Together, the evaluation of binding antibodies provides standardized assessments of immune measurements encompassing concentration (µg/mL), antibody titer (area under the curve, AUC) mean fluorescence intensity (MFI), isotype/subclass, epitope/antigen, avidity, and breadth, with the aim of reconstructing and defining the polyclonal response in individual vaccine recipients with maximum neutralization and antiviral potency [5].

2. PASSIVELY DELIVERED BNABS FOR HIV-1 PREVENTION

A major goal of the laboratory program for clinical studies of passively delivered bnAbs is to determine whether the bnAbs retain their expected neutralizing activity in serum samples collected at various time points post-administration. Serum ID_{50} and ID_{80} neutralizing titers (serum dilutions

inhibiting infection by 50% and 80%) are compared to predicted titers, which are the serum concentration measured in a bnAb-specific binding assay used to assess pharmacokinetics divided by the 50% and 80% inhibitory concentrations (IC_{50} and IC_{80}) of pre-administrated bnAbs (same units for both measurements, *e.g.*, µg/ml). Results have shown excellent agreement for VRC01 [83-85], VRC07.523LS [86] and a triple combination of bnAbs [87], *i.e.*, serum concentration of the bnAbs closely predicts the serum neutralization titers. When combination bnAbs are evaluated, the individual activity of each bnAb pre- and post-administration is quantified using bnAb-specific viruses, whereas their combined activity is quantified using a small panel of viruses known to be sensitive to all three bnAbs [87].

Biological drug products, including monoclonal antibodies, can be immunogenic and induce undesirable anti-drug antibodies (ADA), which may interfere with drug action and hasten drug clearance [88]. ADA is monitored in clinical studies of HIV-1 bnAbs using a multi-tiered approach for their detection, confirmation and detailed characterization [89], including interference with bnAb activity in a functional assay [90]. Dr. Margaret Ackerman's laboratory at Dartmouth conducts assays for ADA detection, confirmation and initial characterization as part of the HVTN and CAVD laboratory programs. Functional assays are performed in Dr. Montefiori's laboratory at Duke for the HVTN and in Dr. Michael Seaman's laboratory at Beth Isreal Deaconess Medical Center for the CAVD.

As mentioned above, the AMP trials of VRC01 for HIV-1 prevention showed for the first time that serum neutralization in the TZM-bl assay could predict antibody-mediated protection against HIV-1 acquisition [19]. They identified a serum neutralization biomarker (ID_{80}= 200) as a surrogate endpoint for benchmarking candidate vaccines and designing future AMP trials [20]. The laboratory program for VRC01 AMP consisted of multiple components with a major focus on an ability to identify a neutralization sieve effect, with the hypothesis that viruses from infections in the treatment groups would be significantly more resistant to VRC01 than viruses from infections in the placebo arm of the trials. Full-length *rev-env-nef* sequences from the earliest HIV-1 RNA-positive plasma samples were obtained using a unique molecular identifier-tagged PacBio sequencing methodology [91]. Transmitted-founder lineage sequences were synthesized, cloned into an expression vector, and sequenced for quality control prior to making Env-psedotyped viruses. Each Env-pseudotyped virus was titrated and assayed three times for accurate measurement of VRC01 IC_{50} and IC_{80} values for use in correlates analyses and the identification of a PT80 biomarker [19, 20]. A complementary genotypic sieve analysis identified Env amino acid sequence features in the VRC01 epitope that associate with prevention efficacy and support the PT80 biomarker [92]. The overall laboratory program for VRC01 AMP provided substantial value and serves as a model for the planned efficacy study of a triple combination of bnAbs.

The design of the pending Combo AMP trial utilizes PT80 derived from the activity of the three bnAbs against the full set of AMP placebo viruses as the most updated estimate of PT80 that is possible at this time [20]. The outcome of the Combo AMP trial, which aims to achieve ~90% prevention efficacy and possibly deliver another viable option for PrEP, will determine how well PT80 based on VRC01 predicts the efficacy of a combination of bnAbs to three different epitopes and where the three bnAbs carry an LS mutation. Because Combo AMP will be evaluated for efficacy in adults in countries where mostly HIV-1 subtypes B and C circulate, bridging studies to determine how well the bnAbs neutralize other major circulating subtypes (*e.g.*, A, D, F, AE, AG, BF) and breast milk transmitted viruses will be necessary to support expanded access of an approved product. Currently, there is a critical shortage of these latter strains of the virus to conduct adequate bridging assessments.

In addition to neutralizing activity, measurements of serum and mucosal mAb concentrations in passively delivered bnAb trials inform decisions on dose, route, and administration schedule and to observe early indications of possible ADA. Qualified and validated anti-idiotypic assays that measure concentrations of administered bnAbs in serum/plasma and mucosal samples are part of the evaluation of phase I and preventative efficacy clinical trials, therapeutic antibody treatment for virus suppression, and immune correlates of protection [20, 93-100]. Pharmacokinetic serum concentrations of VRC01 correlated with the prevention of HIV-1 acquisition [83], providing a benchmark for the level of bnAb concentrations needed for protection. PK analyses are highly sensitive (*e.g.*, the limit of detection/quantitation of the ng/mL or pg/mL range). They are adapted to provide continued support for PK analysis of bnAbs in human clinical trials. For triple mAb combination trials, a high-throughput and sample-sparing assay has enabled simultaneous detection of three different mAbs - a strategy critically important for maximum coverage and potency of neutralization against circulating HIV-1 strains [101].

3. MONITORING FC-MEDIATED ANTIBODY EFFECTOR FUNCTIONS

Optimal Fc features of antibodies may be helpful components of a successful HIV-1 vaccine and passive bnAb strategy. Indeed, neutralizing and FcR-mediated effector functions of antibodies are not always mutually exclusive, and both may contribute to preventing HIV-1 infection. Studies of bnAbs in animal models demonstrate that antibody specificity and Fc-mediated antiviral effector functions of bnAbs contribute anywhere between 21% and 45% to the protective functions [102-104]. Recently, Fc effector functions correlated with protection from simian-human immunodeficiency virus challenge in a nonhuman primate study of a pentavalent vaccine regimen [105]. In addition, certain Fc effector functions may limit founder viruses *in vivo* [106] and increase the ability of bnAbs to eliminate infected cells [107]. As mentioned above, Fc effector functions were associated with the apparent modest efficacy in the RV144 trial [28]. They are also of interest as part of strategies aimed at controlling virus replication [108] and eradicating the latent reservoirs [109] in people living with HIV-1.

3.1. ADCC

One Fc effector function of clinical importance is antibody-dependent cellular cytotoxicity (ADCC). ADCC activity of vaccine-elicited antibodies and bnAbs is assessed in

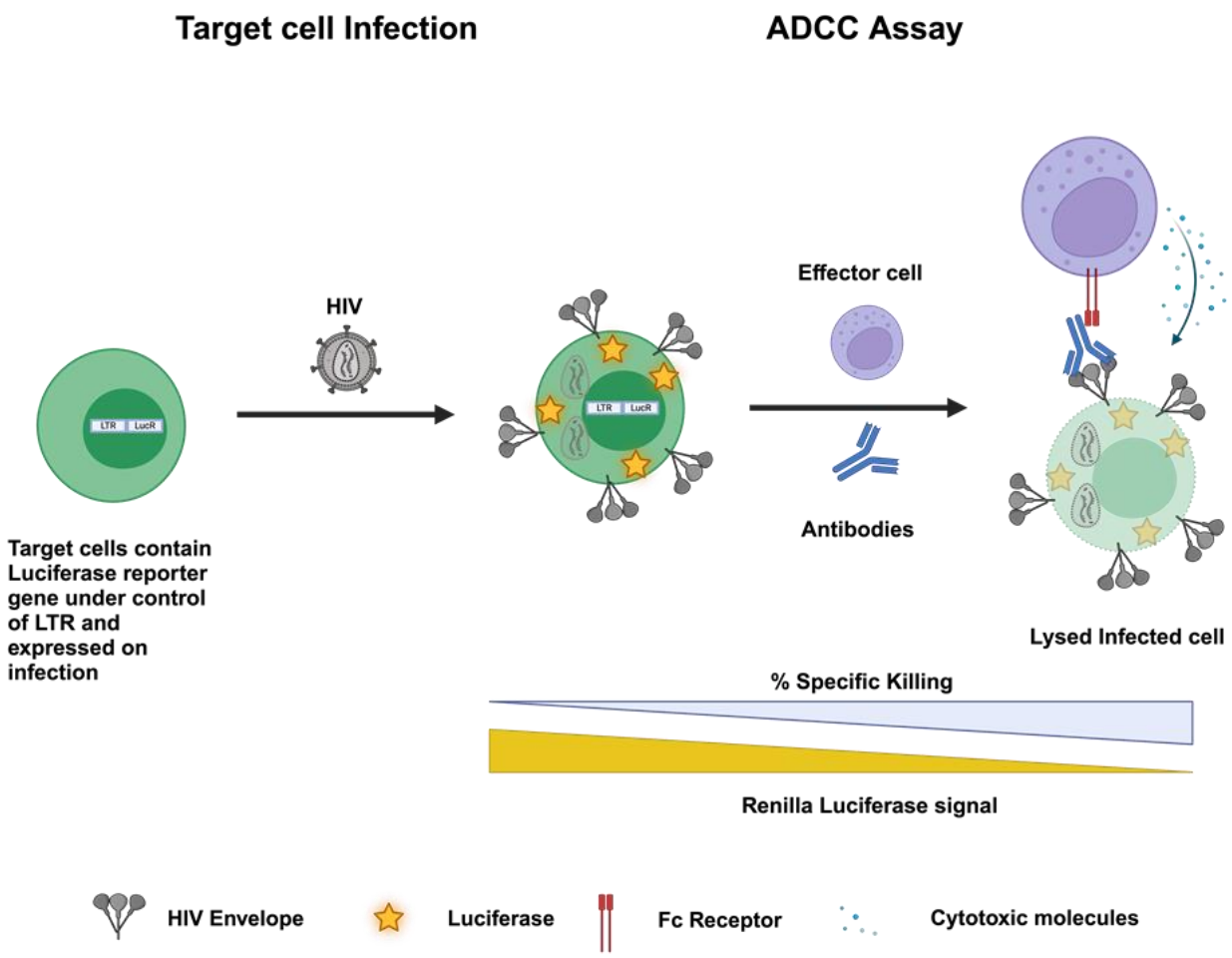

Fig. (3). Schematic representation of HIV-1 ADCC luciferase assay. Target CEM.NKR$_{CCR5}$ cells transduced to contain a Renilla luciferase reporter gene under control of the HIV-1 LTR are infected with HIV-1 viruses. Upon infection, cells express Renilla luciferase and HIV-1 proteins, including Env. Renilla luciferase signal is measured in live cells. When effector cells and HIV-1-specific antibodies are added, lysis of infected cells occurs. This results in a reduction in Renilla luciferase signal, which is proportional to the level of antibody-mediated lysis of infected cells. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

three different types of assays that discriminate activity against different epitopes. One assay utilizes a flow-cytometry-based GranToxiLux ADCC (ADCC-GTL) detection system with gp120-coated target cells to evaluate the presence of antibodies against CD4 inducible epitopes in the C1C2, V2, and V3 regions of gp120. This assay has been described in detail [110] and used to reveal important information on protection from infection in non-human primate studies [105, 111] and in the RV144 trial [28], and in characterizing mAb functions [112-114]. The final read-out is the frequency (percentage) of Granzyme B positive cells reported as percent Granzyme B activity.

Two additional assays, both luminescence-based (ADCC-Luc) [112, 114-116], use target cells infected with either Env.IMC.LucR viruses or Env.IMC viruses (Fig. **3**). Both assays identify the killing of cells expressing membrane-bound Envs when partial or full downregulation of the CD4 takes place, respectively. The final read-out is a reduction in luciferase signal upon incubation of target and effector cells in the presence of antibody. Results are reported as the percentage of specific killings. Our preliminary studies indicate that ADCC observed with these assays correlates with the engagement of NK effector cells. Both ADCC assays have been qualified under GCLP guidelines to define their intra- and inter-assay reproducibility, linearity, limit of detection, and robustness.

To strengthen our assessments of ADCC responses, 29 replication-competent molecular clones encoding different Envs and a Renilla luciferase reporter gene (Env.IMC.LucR viruses), inclusive of subtypes A1, B, C, and CFR01_AE Envs, were characterized using serum samples from people living with HIV-1 before 2010. A subset of seven Env.IMC.LucR viruses were computationally selected as reference strains, referred to as the Fc Historical-panel (Fc H-panel), for assessments of the breadth of ADCC responses [117]. Because a recent report by Mhikze *et al*. [57] indicated increased resistance to bnAbs by recent circulating subtype C Envs, a new panel of Env.IMC viruses expressing the Envs of recently circulating subtype B/F1 or C viruses (referred to herein as Fc Circulating-panel or Fc C-panel) were created using sequencing of viruses in samples from placebo participants who acquired HIV-1 in the recent efficacy trials in

southern Africa and the Americas (HVTN703/704/705). Env sequences were selected based on amino acid sequence diversity in the V1V2, CD4bs, and C1C2 regions of gp120. The new Fc C-panel of IMCs does not contain a Renilla luciferase reporter gene. Instead, the Renilla luciferase reporter gene is integrated with a Green Fluorescent Protein (GFP) reporter gene into a CEM.NKR$_{CCR5}$ target cell line (referred to as D660 cells). This novel aspect complements the previous assay by providing target cells that fully downregulate CD4 expression while maintaining the necessary viability for assay performance. We are currently characterizing a new panel of Envs to identify potential overlaps with the previous Fc H-panel and/or unique aspects, as well as for their susceptibility to ADCC by bnAbs of clinical relevance. Of note, these panels are specific to define the breadth of ADCC responses because we observed a lack of correlation between the neutralizing and ADCC functions [117]. An initial analysis of the ability of 12 clinically relevant bnAbs to mediate ADCC against subtype C Env from before 2010 (historical) and 2016-2018 was recently completed [118]. We found evidence of increased resistance to the V3 bnAb PGT121, where the potency of the bnAb was 50% lower against Envs of recently circulating viruses compared to historic subtype C viruses. In addition, >25% of the Envs were completely resistant to VRC01, 3BNC117, PG9, and PGT121. Ongoing work is utilizing the Fc H- and C-panels to explore possible cooperation of neutralizing and Fc-mediated function for prevention of infection.

3.2. Antibody Dependent Cellular Phagocytosis by Monocytes (ADCP) or Neutrophils (ADNP)

The capacity of specific antibodies to engage effector cells and mediate HIV-specific phagocytosis correlates with protection in clinical and preclinical HIV-1 vaccination studies [33, 105, 119, 120]. Phagocytosis is a process by which macrophages, neutrophils, and dendritic cells destroy antibody-opsonized material and can contribute to the destruction and clearance of viruses or virally infected cells. HIV-1 antibodies that recognize epitopes on HIV-1 virions and engage immunostimulatory Fc receptors on monocytes and neutrophils can mediate the antiviral function of antibody-dependent phagocytosis. ADCP and anti-Env IgG3 breadth correlated with decreased HIV-1 risk in a clinical trial [32]. Similar to the binding assays, panels of recombinant Env proteins are utilized to assess breadth and epitope specificity.

3.3. FcR Binding Assay

Antibody binding to Fc receptors on effector cells is a prerequisite of multiple antiviral functions, including ADCC, ADCP, and antibody-mediated complement deposition, highlighting the need to better understand antibody and FcR biology to identify protective antibody-mediated immunity. Moreover, host genetic differences in FcR can influence vaccine efficacy [32, 121, 122]. A high-throughput binding assay measures antigen-specific binding antibody interactions with FcRs (*e.g.*, FcRγRI, FcRγRIIa, FcRγRIIIa, FcRα) in systemic and mucosal samples uses biotinylated FcR proteins tetramerized with phycoerythrin (PE) as a detection reagent [123-125]. Biophysical characterization of antibody interaction with FcRs by Biolayer Interferometry (BLI) or Surface Plasmon Resonance using standardized/qualified methods enables further insights into the strength of interactions that modulate the inflammatory and antiviral responses to HIV-1.

4. MUCOSAL ANTIBODIES

Evaluation of the mucosal antibody response to vaccination can provide insights into the antibody specificities, forms, and functions necessary to prevent virus infection at primary sites of HIV-1 exposure. Binding antibodies (*i.e.*, IgM, IgG, IgA, SIgA, Ig subclasses) in all mucosal types (*i.e.* cervical/vaginal, rectal, saliva, semen, breastmilk) are measured by BAMA across a broad panel of HIV-1 Env antigens while controlling for total antibody in the specimen [126-128]. These analyses shed light on the specificities and functions (*e.g.*, phagocytosis, virion capture) of antibody responses. A meta-analysis across human clinical trials to evaluate genital mucosal antibodies compared to systemic responses elucidated the impact of vaccine type on antibody dynamics, including isotype, specificity, breadth, magnitude, and durability and the continued difficulty in eliciting mucosal HIV-1-specific IgA by vaccination [129]. The ultimate success of bnAb-inducing vaccine regimens to prevent acquisition at the portal of entry may depend on sufficient concentrations of bnAbs and potentially antibodies such as SIgA at the mucosa.

CONCLUSION

The HIV-1 vaccine field has made remarkable progress in designing germline-targeting immunogens that induce early precursors of multiple epitope classes of bnAbs, and efforts to mature these responses are gaining traction. Specialized neutralizing and binding antibody assays designed to detect early precursors of bnAbs and track their maturation are facilitating these efforts by enabling progress to be monitored serologically as a complement to phenotypic and molecular interrogations at the B cell level. The availability of diverse panels of reference viruses and Env antigens further strengthens these efforts. PT80=200 from the VRC01 AMP trials provides an important benchmark for success but also represents a high bar that will be difficult to achieve. Vaccine induction of virus-specific T cells and antibodies with non-neutralizing antiviral effector functions could potentially reduce the threshold of neutralizing antibodies required for protection [130]. Thus, multiple antibody and T cell functions may be essential components of a collaborative system of protective immunity against HIV-1 acquisition that is important to monitor in the preclinical and clinical phases of vaccine development.

AUTHORS' CONTRIBUTIONS

It is hereby acknowledged that all authors have accepted responsibility for the manuscript's content and consented to its submission. They have meticulously reviewed all results and unanimously approved the final version of the manuscript.

LIST OF ABBREVIATIONS

AMP	=	Antibody Mediated Protection
ADA	=	Anti-Drug Antibodies
BAMA	=	Binding Antibody Multiplex Assays

BnAbs	=	Broadly Neutralizing Antibodies
MPER	=	Membrane Proximal External Region
PrEP	=	Pre-Exposure Prophylaxis
GCLP	=	Good Clinical Laboratory Practices
RLUs	=	Relative Luminescence Units
MFI	=	Median Fluorescence Intensity
FcR	=	Fc Receptor

CONSENT FOR PUBLICATION

Not Applicable.

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None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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