Engineering broadly neutralizing antibodies for HIV prevention and therapy

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Abstract

A combination of advances spanning from isolation to delivery of potent HIV-specific antibodies have begun to revolutionize understandings of antibody-mediated antiviral activity. As a result, the set of broadly neutralizing and highly protective antibodies have grown in number, diversity, potency,a and breadth of viral recognition and neutralization. These antibodies are now being further enhanced by rational engineering of their anti-HIV activities and coupled to cutting edge gene delivery and strategies to optimize their pharmacokinetics and biodistribution. As a result, the prospects for clinical use of HIV-specific antibodies to treat, clear, and prevent HIV infection are gaining momentum. Here we discuss the diverse methods whereby antibodies are being optimized for neutralization potency and breadth, biodistribution, pharmacokinetics, and effector function with the aim of revolutionizing HIV treatment and prevention options.

1. Introduction

Antibodies have demonstrated therapeutic utility since at least the 1890s in the form of “serum therapy” used to combat infectious diseases such as diphtheria, tetanus, [influenza](#), measles, and polio. Although the first clinically approved monoclonal antibody (mAb), palivizumab [1, 2], similarly found utility in an infectious disease setting (RSV), the majority (28 out of 30) of current therapeutic mAb therapies have been developed for non-infectious applications in allergy, autoimmune diseases, and cancer. The demonstration of both clinical safety and efficacy of this treatment modality time and again across diverse disease settings has led to rapid growth in therapeutic mAb development in recent years. This renewed interest in antibody therapies has led to rapid technological advances in the isolation and characterization of mAbs, [ushering](#) in an era in which mAb engineering strategies are routinely used to translate basic understandings of antibody feature contributions into more efficacious and creative treatment and [prevention](#) strategies.

Against HIV, anti-viral antibodies have demonstrated [the capacity](#) to reduce viremia and protect from infection in several animal models [3-13] and in humans [14-16]. [Humoral responses to HIV may be classified as non-neutralizing, neutralizing, or broadly neutralizing based on their ability to inhibit viral attachment and cell entry via binding to the HIV envelope glycoprotein \(Env\). The extensive pathogenic diversity and rapid evolution of HIV poses a considerable challenge to the *natural* development of neutralizing antibodies \(NAbs\) before establishment of a permanent viral reservoir.](#)

[Although the two HIV types \(HIV-1 and HIV-2\) share ~30-60% genetic similarity, they differ greatly in their epidemiology: HIV-1 dominates public attention as the most widespread and increasingly prevalent global health issue, whereas HIV-2 predominates in West Africa and is \[currently\]\(#\) declining in prevalence \[17, 18\]. Furthermore, HIV-2 infection progresses to AIDS much more slowly than HIV-1 \[19, 20\] and is significantly less transmissible \[21\]. In addition to stronger virus-specific T-cell mediated immune responses \[22-24\], cellular resistance to infection \[25\], lower viral replication kinetics \[26, 27\], and an immune protective function of part of the HIV-2 envelope \[28, 29\], increased and broader NAb responses are implicated in the significant viral control and slower disease progression found in the majority of HIV-2 infected individuals \(recently reviewed in \[30\]\). From HIV-1 infection, broadly neutralizing abs \(bNAbs\) may appear after several years of persistent antigen exposure in a subset of patients with high viral load and progressive disease \[31\], at which point they are too late to offer any clinical benefit. In contrast, HIV-2 infection frequently results in the development of bNAb responses \[32-36\], and many infected individuals maintain low viral loads and normal CD4 T cell counts without antiviral](#)

therapy [19, 37-39]. In this review, we focus on engineering bNAbs against HIV-1 infection but much can be learned from natural HIV-2 humoral immunity. At the level of the infected individual, HIV-2 Env evolves at equivalent/faster rates than HIV-1 [40, 41], likely due to selective pressure from NAb responses [42]. Antigenic differences allowing for the greater generation of NAb responses in HIV-2 as compared to HIV-1 include greater stability of the Env trimer [43], less glycan shielding and a more 'open' conformation allowing for greater accessibility of NAb epitopes [36, 44], and greater structural and functional constraints to diversity in some NAb epitopes as compared to HIV-1 [45]. Differences in humoral responses to HIV-2 infection as compared to HIV-1 infection include 1) an increased frequency of both IgG and IgA responses [32, 46-48], 2) a predominance of IgG1 & IgG3 subclasses [48] (whereas the more inert IgG2 & IgG4 subclasses are additionally found in viremic HIV-1 infected individuals [49]), and 3) a greater contribution of antibody-dependent complement-dependent cytotoxicity to antiviral activity [50].

Despite the apparently greater challenges to mounting a natural antibody response to HIV-1 with high neutralization and effector capability, engineered bNAbs may similarly offer significant therapeutic potential to HIV-1 infected individuals. Rapid progress in screening HIV-1-infected donors for serum neutralizing activity [51-53] and in efficient antigen-specific B-cell sorting [54, 55] has led to the discovery of new, highly potent bNAbs [54, 56-65], as well as the structural definition of major sites vulnerable to neutralization on HIV-1 Env [60, 61, 66-81]. HIV-1 Env assembly entails heterodimerization of gp120 and gp41 subunits to form gp140 protomers, and trimerization of gp140 protomers (Figure 1A). Trimeric Env is inherently unstable, heavily glycosylated, expressed at low levels on the viral surface, and provides limited access to functionally critical epitopes [82, 83], features that contribute to viral fitness, immune escape, and the propensity to generate non-neutralizing antibody responses. Despite extensive viral diversity in Env sequences, bNAbs target conserved epitopes (Figure 1B), often involving both amino acid residues and/or glycosylation motifs, shared across several viral strains. Recent advances in next generation sequencing and antibody isolation have allowed researchers to better understand and identify both bNAb structural features contributing to potency and more broadly conserved Env epitopes targeted by bNAbs. These findings are catalogued on two publicly available databases: CATNAP (LANL) [84] and bNAber [85]. bNAb target epitopes comprise a broad continuum along the Env glycoprotein [86] but are commonly divided into 5 domains including 1) the trimer apex and variable loops V1, V2, and V3, 2) the V3 glycan supersite at Asn332, 3) the CD4 binding site (CD4bs), 4) the gp120-gp41 interface, and 5) the gp41 membrane-proximal external region (MPER) (Figure 1C).

Shared features of bNAbs isolated from [HIV-1](#)-infected individuals include high levels of somatic hypermutation [87-89], with 40-100 nucleotide mutations present in the heavy chain variable region (VH) alone [57, 58, 61, 72, 89-92], high levels of insertions/deletions [93], and long CDR-H3 regions which facilitate penetration of the Env glycan shield and access to functionally conserved regions [67, 71]. Whether these and other unusual features are generally required for broad neutralization or simply arise from extended periods of antibody maturation and selection is currently unknown, but has inspired rational protein engineering efforts based on sequence information from germline ancestors and clonal families of bNAbs. Generally, bNAbs exhibit characteristics reflective of natural trade-offs between potency, breadth, and specificity.

2. HIV-specific mAb therapeutic applications

HIV-specific mAbs have potential clinical roles in prevention, therapy, and even functional cure of HIV infection. Each of these applications may require antibodies optimized for different properties based on the different mechanisms of action that might be most effective in each setting.

2.1 Prevention of Infection

Naturally-raised neutralizing antibodies have been implicated in preventing infection in HIV-exposed but uninfected individuals [94-97] and in reducing the risk of mother-to-child transmission (MTCT) [98-102]. Beyond neutralization capacities, effector functions of antibodies have correlated with protection or reduced viral loads in vaccine trials in macaques [103-107] and reduced risk of infection in human VAX004 and RV144 trials [108-110]. Direct evidence for bNAb protection from acquisition of infection has been limited to pre-clinical models, which demonstrate that passive administration of bNAbs provides sterilizing immunity against simian HIV (SHIV) infection in macaques [5-7, 10, 111]. However, *ex vivo* studies of the neutralization sensitivity of human infant transmitted/founder strains to bNAbs have demonstrated encouraging results for MTCT prophylaxis [112-114], including the finding that the combination of PG9 and NIH45-46([G54W](#)) neutralized all tested viruses from almost 90% of mother-infant pairs [114], and a Phase I clinical trial to evaluate the use of VRC01 in HIV-1 exposed infants is currently recruiting (clinicaltrials.gov identifier: NCT02256631).

2.2 Therapeutic Suppression of Viremia

Particular features of naturally-raised anti-HIV antibodies are associated with spontaneous control of viremia without antiretroviral therapy (ART) in a subset of infected individuals known as

elite controllers [115-117]. Passively administered bNAbs have resulted in suppression of viremia after established infection in macaques [106, 118], humanized mice [119-123], and humans [14-16]. While clinical trials of first generation bNAbs, F105, 2F5, 2G12, 4E10, KD-247, and p2G12, have been completed in humans with largely underwhelming results of transient decreases in viral load [14, 15], the recent surge in identification/isolation of more potent bNAbs, combined with impressive demonstrations of protective effects in preclinical animal models, has inspired the return of anti-HIV mAbs to clinical trials with several currently recruiting to evaluate candidates 3BNC117, 10-1074, PG9, and VRC01 (clinicaltrials.gov).

Most recently, the results from the first Phase I dose-escalation studies of CD4bs-specific bNAbs VRC01 [124] and 3BNC117 [16] in humans have demonstrated clinical safety and, in the case of 3BNC117, the capacity to transiently reduce viral load for as long as therapeutic concentrations were maintained in serum in a dose-dependent manner. High doses resulted in up to 2.5log10 reductions in viral load. Additionally, the single individual who did not respond was infected with a virus resistant to 3BNC117. The single IV dose of 3BNC117 exerted selective pressure on patient viral populations and resulted in the development of highly resistant strains in some, but not all individuals, as demonstrated by 3BNC117 sensitivity testing of patient viral isolates and sequence analysis of viral Env. This landmark study demonstrates the significant therapeutic potential of bNAbs for treatment of HIV-infected individuals, although combinations of mAbs to avoid resistance or adjunctive therapy with ARV [\[106, 118, 119, 125\]](#) are expected to be required.

Additionally, passively administered anti-HIV antibodies can demonstrate indirect 'vaccine-like effects' whereby stimulation of the endogenous host immune response contributes to antiviral activity lasting well beyond the treatment period itself (reviewed [126]). In macaque studies of SIV and SHIV infection, SIV-/SHIV-IG treatment not only delayed disease onset and increased survival rates, but also accelerated *de novo* production of autologous anti-SIV antibodies [127, 128] and elevated virus-specific CD4+ and CD8+ T cell responses during both acute and chronic infection phases [129]. Similarly, administration of monoclonal anti-HIV bNAbs after SHIV infection to macaques also correlated with a slight increase in neutralizing antibody titers and significantly improved functionality of Gag-specific CD4+ and CD8+ T cell responses, with a subset of animals maintaining long-term virologic control in the absence of further mAb infusions [106]. While viral escape from single bNAb treatment does occasionally occur in macaques, as demonstrated by studies administering 3BNC117 and 10-1074 [118], single mAb treatment in humanized mice invariably leads to rapid viral escape from mAb neutralization [119, 125]. A recent critical study elucidated these results by demonstrating that

autologous host humoral responses present in macaques (but impaired in humanized mice) contribute to the control of viremia by synergizing with passively administered bNAbs to prevent the emergence of viral escape variants [130]. Thus, therapeutic administration of bNAbs after established HIV infection may suppress viremia through both direct antibody-mediated functions and indirect stimulation of endogenous antiviral immune responses.

2.3 Functional Cure: Persistent surveillance and eradication of latent reservoirs

As opposed to sterilizing protection to prevent establishment of viral reservoirs at the time of initial viral exposure, a cure for chronic HIV infection requires a durable therapeutic to continuously surveil and destroy newly activated reservoir cells and/or clear or compromise nascent virions [131]. The only example of complete viral eradication thus far has been the Berlin patient, a formerly HIV-infected individual with acute myelogenous leukemia who underwent bone marrow/stem cell transplantation from a donor lacking expression of viral coreceptor CCR5 [132]. Less drastic approaches that preserve autologous immunity may instead implement mAbs to target viral reservoirs: 1) “kick and kill” strategies employ a combination of viral inducers to activate viral reservoirs and bNAbs to eliminate newly activated cells [131, 133], and 2) gene delivery strategies induce long-lasting expression and production of mAbs to continuously protect against newly activated reservoir cells and virion release [134]. For these strategies, understanding the dynamics of viral Env epitope expression on the surface of latently infected cells will be critical: current studies suggest that early in infection prior to virion release, cells will more likely express trimeric Env proteins [135] whereas later in the virus infection cycle, cells will more likely express monomeric gp140 and gp41 stumps as the dissociated remnants of viral Env [133]. Thus, neutralizing Abs may be best suited for acutely infected and recently reactivated cells and non-neutralizing Abs recognizing gp41 epitopes for latent, chronically infected cells. Furthermore, viral reservoirs may exist in multiple tissues [136], and concerns regarding compartmental differences in innate immune cell populations and access to immunologically privileged sites [133] may require the addition of nonnative functions to bNAbs to fully eradicate reservoirs.

Engineering strategies to enhance the clinical utility of bNAbs in each of these indications will be discussed according to approaches aimed at the different structural aspects of antibody activity: 1) Fv engineering to improve neutralization potency and breadth and to decrease polyreactivity and viral escape ([Table 1](#)), 2) Fc engineering to improve half-life, biodistribution, and recruitment of innate immunity ([Table 2](#)), and 3) combination with additional functional

agents ([Table 3](#)), such as effector recruitment signals (T-cell engaging scFvs), and even cellular therapies (Chimeric Antigen Receptors). Finally, delivery strategies that can be applied to any engineered bNAb to further improve bioavailability and durability of response will be discussed.

3. **Fv engineering:**

A recent study detailing the incomplete neutralization profiles of several bNAbs [137] reveals shortcomings in Fv regions of naturally elicited bNAbs that may be improved through engineering strategies ([summarized in Table 1](#)). For prevention of infection, broad and potent neutralization are particularly crucial to prevent establishment of a viral reservoir. After infection, additional measures to avoid viral escape are necessary to suppress viremia therapeutically. Towards functional cure, identification of the most prevalent epitopes on latent reservoir cells is most critical, but additional engineering to enhance potency may be necessary to target the Env expressed on reactivated, latently infected cells. In all of these cases, engineering strategies to reduce the polyreactivity common to many bNAbs will improve therapeutic efficacy and reduce the risk of adverse reactions.

3.1 To improve Potency & Breadth

Protein engineering strategies to improve the breadth and potency of bNAbs (reviewed in [138]) include both combinatorial directed evolution techniques and rational computational strategies. Early directed evolution of bNAb b12 through phage display enhanced affinity nearly 400-fold [139] and demonstrated that improved affinity could lead to increased breadth of neutralization [140]. This study demonstrated that affinity can in some cases be used as a surrogate marker for both potency and breadth in evolutionary strategies, suggesting the value of affinity maturation as a generalizable strategy for enhancing bNAb activity. In another approach, the neutralization breadth of the HIV-1 m9 scFv was evolved from a previously isolated X5 bNAb from phage display libraries by selecting for binders to sequentially changing antigens [141].

Computational strategies instead use information provided by both clonally related bNAbs and more extensive antibody databases and molecular modeling to inform mutational choices. The variable fragment (Fv) of antibodies consists of complementarity-determining residues (CDRs) involved in antigen-binding specificity and framework residues (FWRs) contributing to antibody structure. Rational protein engineering approaches to enhance potency and breadth of Env binding commonly manipulate CDR residues to enhance stability, conformation, orientation, energetics, or kinetics of Ab:antigen interactions. For example, stabilizing the CDR3 of the heavy chain (CDR-H3) of bNAb PG9 in its active conformation led [to the](#) increased potency and

breadth of [engineered](#) variant PG9_N100(F)Y, which neutralizes diverse PG9-resistant HIV strains, some of which lack the Env N160 glycan critical for PG9 binding [142]. Rational mutations to improve hydrophobic interactions [65, 143], leverage glycan contacts [144], and to avoid steric clashes [144] between antibody and antigen have similarly generated VRC01-class Ab variants with increased potency and breadth and have resulted in neutralization of strains resistant to the original antibody. Combining CDR features of bNAbs targeting similar epitopes has conferred increased breadth of neutralization and accommodation of glycan heterogeneity and/or proximal membrane lipids to antibody hybrids of 10-1074 & PGT121 [60], PG9 & PG16 [69], and 4E10 & 10E8 [145]. Although often overlooked in antibody-antigen interactions, FWRs may present another strategy through which to enhance antibody Fv's, as some FWR mutations directly contribute to breadth and neutralization [89, 146].

An alternative strategy replaces the Fv region of an antibody entirely with the extracellular domain of CD4, the primary receptor of HIV-1 Env. These CD4-Ig immunoadhesin molecules achieve broad neutralization, irreversible inactivation of Env, and selection for less-fit escape variants with impaired receptor binding [147-150] but suffer from a lower affinity for Env compared to bNAbs [151] and a simultaneous capacity to promote infection at subneutralizing concentrations (undetermined mechanism) [152]. Nevertheless, CD4-Ig molecule PRO542 has demonstrated safety and efficacy (80% response rate, $\sim 0.5 \log_{10}$ reduction in viral load 4-6wks post-treatment) as salvage therapy in advanced HIV-1 disease [153] and a [recently](#) improved version fusing CD4-Ig with a CCR5 mimetic peptide at the C-terminus of the Fc, named eCD4-Ig, has demonstrated unmatched neutralization breadth (including strains resistant to CD4bs bNAbs NIH45-46, VRC01, 3BNC117) and potency thought to be due in part to higher avidity from the CCR5 mimetic, a decreased ability to promote infection, and enhanced antibody-dependent cellular cytotoxicity (ADCC) [154]. Significantly, AAV delivery of eCD4-Ig to rhesus macaques protected all inoculated macaques from multiple infectious doses, lasting for at least 34 weeks after inoculation. Thus, eCD4-Ig represents one of the broadest "bNAbs" tested to date and may prove beneficial in combination therapy with other bNAbs or as sole AAV therapy if anti-eCD4-Ig responses, or other aspects of dosing subjects with recombinant CD4 receptor, do not preclude its use.

3.2 Restricting viral escape

Even with the engineered improvements in potency and breadth of VRC01-class engineered antibody NIH45-46(G54W), some HIV-1 clones are naturally resistant [143] and escape mutants can emerge during exposure [125]. Rational engineering of NIH45-46(G54W) based on the

structure of the NIH45-46—gp120 complex and Env sequences of NIH45-46-resistant viral strains resulted in variants 45-46m2 and 45-46m7 [144]. Whereas viral escape mutants in mice treated with NIH45-46(G54W) exhibited mutations anywhere along the highly conserved N/DNGG (aa279-282) consensus sequence identified as involved in resistance to this Ab [155], escape mutants from mice treated with the enhanced variants 45-46m2 and 45-46m7 were limited to substitutions to introduce a potential N-linked glycosylation site (PNGS) at N279 that introduces a fitness cost that may be sufficiently severe to account for its rare or nonexistent presence in natural HIV-1 strains catalogued in the Los Alamos database [144]. By restricting the pathways for HIV-1 escape with the new 45-46m2/m7 variants and imposing a fitness cost for escape mutations, the authors demonstrated control of viremia in humanized mice using only three antibodies—45-46m2, 45-46m7, and 10-1074—targeting only two epitopes. Thus, protein engineering strategies to restrict viral escape can allow for better viral control with fewer mAbs.

A second strategy to combat resistance involves the identification and combination of mAbs with complementary resistance patterns, where mutations leading to viral escape from one mAb render it more susceptible to the complementary mAb. Studies investigating the *in vitro* neutralizing activities of combinations of 2-4 mAbs targeting four distinct epitopes (CD4bs, V1V2-glycan, V3-glycan, gp41MPER) across a panel of 125 Env-pseudotyped viruses found that combinations of bNAbs with complementary neutralization profiles recognizing distinct epitopes resulted in improved neutralization breadth closely predicted by an additive effect model [156]. At 50% inhibitory concentration (IC₅₀) cutoffs of 1 μg/mL per antibody, combinations of two mAbs neutralized 89-98% of viruses and combinations of three neutralized 98-100% of viruses. Statistically significant, albeit weak, synergy was further observed in 15 out of 22 mAb combinations, consistent with earlier reports combining earlier generation bNAbs [157, 158]. By targeting multiple epitope specificities, combinations of mAbs increase the breadth of viruses neutralized and may thereby prevent resistance by outpacing viral evolution and escape.

In addition to targeting various Env epitopes, therapeutic anti-HIV mAbs may target host cellular receptors necessary for viral entry (recently reviewed in [159]). Ibalizumab (iMab), a humanized IgG4 against the CD4 receptor, has demonstrated broad inhibition against a panel of >100 HIV-1 Env pseudoviruses *in vitro*, inhibiting 92% of viruses, but has substantially lower potency than bNAbs PG9 or VRC01. Although the mechanism of action remains uncertain, recent single-molecule force spectroscopic analysis of the CD4-gp120 interaction suggests that gp120 binding to CD4 induces a mechanical extension of CD4 domains 1 and 2 that is inhibited by iMab [160]. iMab is currently in phase 3 clinical trials for HIV-1 infection and has received orphan drug designation from the US FDA Office of Orphan Products Development. The

predominant resistance mechanism against iMAb entails the loss of V5 PNGS which enhance sensitivity to neutralization by VRC01 [161], and other resistant variants have demonstrated enhanced sensitivity to soluble CD4 (sCD4) [162]. Thus, combining iMAb with CD4bs-targeting bNAbs or sCD4 may also prevent viral escape.

Similarly, PRO140, a humanized IgG4 against the CCR5 coreceptor, has demonstrated broad anti-HIV-1 activity in PBMC and macrophage neutralization assays against R5- and dual-tropic viruses [163]. PRO140 acts noncompetitively to allosterically inhibit viral attachment [164] and is currently in phase 2b studies. [Viral resistance to therapy develops less rapidly for PRO140 compared to traditional ARV: R5 viral susceptibility to PRO140 remained intact following 3 weeks of subcutaneous monotherapy \[165\] and two weeks after single IV dose of PRO140 \[166\].](#) Furthermore, bispecific IgG combining the specificities of bNAbs with iMAb or PRO140 exhibited highly synergistic effects [167]. One tetravalent, bispecific CCR5 antibody blocking two alternative docking sites of R5-tropic HIV strains on the CCR5 coreceptor has demonstrated 18-57 fold improved potency with one combination demonstrating antiviral activity against virus strains resistant to each parental Ab alone [168].

3.3 To Decrease Polyreactivity and Improve Pharmacokinetics

Polyreactive/autoreactive antibodies have been associated with HIV-1 infection for [more than 25 years](#) [169-171], and higher frequencies of polyreactivity are found among bNAbs compared to non-neutralizing Abs [172]. Importantly, multiple studies have reported [that](#) increased polyreactivity and/or reduced solubility and increased aggregation propensity can accompany increasing breadth/potency [65, 173, 174]. [Polyreactivity is correlated with increased rates of clearance *in vivo* \[175\].](#) Efforts to combat polyreactivity and aggregation propensities that could lead to polyreactivity include isotype switching, inserting N-linked glycosylation sites, and reducing the number of surface hydrophobic residues [138]. In engineered variants of NIH45-46 and VRC07-523 [65, 143], a G54W mutation enhanced potency but also increased polyreactivity. Substitution to histidine, which was observed in clonal relatives and thereby likely acceptable to Ab function, decreased polyreactivity and improved pharmacokinetics while maintaining the improved potency of G54W [65]. Sequence [and](#) structural analysis of clonal relatives of 10E8 similarly led to new variants with equivalent potency and nearly 10-fold increased solubility [176].

4. Fc Engineering

Antibody Fc's engage a wide range of soluble and cellular receptors (recently reviewed in [177]) with varying preferences dependent upon antibody isotype, subclass, and glycosylation

status. By binding to transport receptors, the antibody Fc directs the trafficking of antibodies and immune complexes and determines both the serum half-life and biodistribution of therapeutic mAbs. By binding to Fc Receptors (FcRs) on innate immune cells or complement receptors (such as C1q), the Fc serves as a mediator between host innate and adaptive immune responses and stimulates effector cells to contribute not only to direct antibody-mediated responses such as Ab-dependent cellular cytotoxicity (ADCC), Ab-dependent cellular phagocytosis (ADCP), and antibody-dependent complement-dependent cytotoxicity (ADCC), but also to the generation of durable endogenous adaptive immunity [126]. Thus, engineering the Fc domains of HIV-specific antibodies (reviewed in [178] and [179]) to enhance half-life, biodistribution, and effector functions can significantly improve bNAbs' clinical efficacy ([summarized in Table 2](#)).

4.1 To increase half-life

Most clinical mAbs are of the IgG isotype (Figure 2), which can interact with the neonatal Fc Receptor (FcRn) in a pH-dependent manner to allow for transport of maternal antibodies to fetuses *in utero* and recycling of antibodies after internalization in numerous cell and tissue types. FcRn binds IgG tightly in acidic endosomal vesicles, but weakly at the neutral cell surface where it is released [180]. FcRn binding can additionally direct antibody transport across epithelial surfaces [181, 182] or immune complex targeting to lysosomes for degradation [183]. A recent key study demonstrated that anti-HIV bNAb variants engineered for enhanced FcRn binding exhibited longer serum half-life, enhanced localization to and persistence at mucosal epithelial surfaces, and ultimately superior protection from mucosal intrarectal SHIV challenge in macaques [181]. In addition to protecting from mucosal transmission, FcRn-binding enhanced variants may prove especially useful in MTCT for *in utero* prophylaxis to deliver bNAbs to the fetus.

Several human IgG Fc variants have been developed to enhance FcRn binding at acidic pH and maintain no or moderate binding at neutral pH [184-186] by targeting Fc residues in the CH2 and CH3 domains responsible for FcRn binding [187, 188]. While these domains are physically distinct from the CH1 and hinge regions bound by FcγRs for effector function, a recent study examining four human IgG1 variants engineered for enhanced FcRn binding found that in most cases, Fc engineered mutants bound human C1q and FcγRs less strongly than the wild type IgG1 and demonstrated varying abilities to induce ADCP and ADCC *in vitro* [189]. The authors noted that differences in glycosylation were found between WT and mutant variants, even when mutations were introduced distally from the N297 glycosylation site. Thus, mutations at the CH2-

CH3 interface may impact the glycosylation profile and/or flexibility of the domains and thereby directly affect interaction with C1q and FcγRs to explain the unpredictable changes in effector functions. This study suggests that Fc engineering for enhanced binding to FcRn may indirectly affect FcγR binding and effector functions, so mAb engineering to optimize mutations to balance increased half-life and clinical efficacy, as has been done for ADCC [190], may be critical.

4.2 To improve mucosal immunity

In addition to IgG, IgA is found at mucosal surfaces through binding of its Fc to polymeric IgA Receptors (pIgR). The role of IgA in HIV infection remains controversial [191], with findings of HIV-specific mucosal IgA in exposed but uninfected subjects [94-97] supporting a potentially protective role of mucosal IgA in HIV infection while associations of HIV-specific plasma IgA with increased risk of infection in a vaccine trial [110] suggest a negative role in protection. Passive transfer studies in macaques allowing for more systematic and controlled evaluation of antibody Fc/dose/localization and route of viral challenge clarify the protective potential of mucosal IgA: intrarectal administration of dimeric IgA1 (dIgA1) afforded greater protection from intrarectal viral challenge than dimeric IgA2 or IgG1 bearing the same neutralizing Fv [192], and the combination of intravenous IgG1 with mucosal administration of dIgA1 demonstrated superior protection to either antibody alone [193]. Thus, in addition to the development of bNAbs as FcRn-binding enhanced IgG1 variants, formulation as IgA (Figure 2) may prove beneficial to protect against mucosal infection.

For mucosal immunity, dimeric IgA (dIgA) binds to the polymeric Ig receptor (pIgR) for transport to mucosal surfaces where it is released as secretory IgA (sIgA) [177]. The localization and polymeric tendencies of IgA enable it to aggregate and trap virions at mucosal sites [194, 195], with enhanced inhibitory effects observed from bNAbs expressed as dIgA compared to monomeric IgA (mIgA) on mucosal transmission of HIV in humanized mice [196]. Successful recombinant protein engineering of mIgA [197], dIgA [198], sIgA [199], and a stabilized IgA2m(2) allotype [200] has been described and avails the opportunity to better understand the protective mechanisms of IgA variants when delivered intravenously vs. mucosally. Further engineering efforts to enhance binding to activating FcαRs to increase ADCP or to polymeric Ig receptor (pIgR) to facilitate excretion of HIV from mucosal lamina propria through transcytosis [201] could additionally enhance protection of bNAbs re-engineered as IgA.

4.3 To enhance effector functions

Antibodies can engage a wide range of innate immune cells to exert antiviral activity through ADCC, ADCP, and ADCDC. Elevated levels of ADCC-inducing Abs correlate [positively](#) with spontaneous control of HIV without ARV in elite controllers [117] and reduced mortality in infant cases of MTCT when passively acquired through breast milk [202] [and](#) inversely with rate of progression to AIDS [203-206] and infection risk in the VAX004 vaccine trial [109] and among individuals with lower IgA responses in the RV144 trial [110]. Similarly, evidence [of a role for](#) ADCP in controlling HIV disease progression has been documented [207-209] and higher antibody phagocytic capacities have been found in HIV elite controllers compared to chronic progressors [115]. The role of antibody-dependent CDC is [less well](#) defined since initiation of the complement cascade may occur in the absence of antibody, making it difficult to separate ADCDC from CDC present in HIV-infection [210]. However, ADCDC has been associated with decreased viral load [211] and neutralization-independent protection from heterologous virus challenge in primary infection [212].

Through antibody effector functions, *non-neutralizing* antibodies may also derive therapeutic value and have been demonstrated to reduce viral load upon topical vaginal application in macaques challenged with Simian/human immunodeficiency virus (SHIV), although no prevention of SHIV acquisition was seen in these animals [213]. Furthermore, a landmark study found that the *in vivo* protective activity of anti-HIV bNAbs more precisely correlates with their ability to engage activating Fc-gamma [receptors](#) (Fc γ Rs) than their *in vitro* neutralization activity [214]. Thus, as bNAbs exert their *in vivo* efficacy through Fc-mediated effector functions, [an](#) opportunity arises to [engineer existing](#) bNAb [Fc](#) regions [to enhance](#) binding to activating Fc [receptors](#) or to skew towards particular Fc-mediated responses.

4.3.1 IgG Subclass

IgG antibodies dominate the anti-HIV response and are responsible for [the](#) effector functions ADCC, ADCP, and ADCDC. The relative prevalence of IgG subclasses IgG1-IgG4 in plasma correspond to their numbering, but each subclass' distinct profile for FcR binding determines its relative contribution to effector functions [215, 216], with IgG1 and IgG3 preferentially engaging activating FcRs for ADCC, IgG2 for ADCP, and IgG4 better engaging inhibitory receptors. [By harnessing](#) these natural variations in effector function preferences, IgG subclass switching has been demonstrated to alter the effector functions and *in vivo* efficacy of [anti-HIV mAbs in mouse models](#) [214], [as well as in numerous other settings](#).

Particular subclass profiles have been associated with viremic control in natural infection: elite controllers tend to exhibit higher IgG1 titers to p24 and gp120 and higher IgG3 titers to Env

gp120 compared to chronic progressors [116], corresponding to increased engagement of activating FcRs for ADCC. Similarly, vaccine trials have also provided associations of particular antibody subclass profiles with reduced risk of infection. RV144 and VAX003 are two different vaccine trials that included administration of the bivalent recombinant gp120 AIDSVAX B/E protein. Both trials elicited gp120-specific antibodies, but poor antibody neutralization activity [217] and negligible cytotoxic T cell responses [218]. However, the RV144 vaccine regimen successfully, albeit moderately, reduced the risk of infection among vaccinees [219]. A recent study demonstrated that antibodies induced by the protective RV144 trial were capable of eliciting multiple effector functions and included induction of IgG3 Abs, whereas VAX003-induced antibodies were more 'monofunctional' and skewed towards the more inert IgG2 and IgG4 subclasses [220]. Even though both trials elicited humoral responses largely composed of IgG1, depletion of IgG3 from RV144 samples significantly reduced ADCC and ADCP activity, and depletion of IgG4 from VAX003 samples significantly increased ADCP activity and trended toward increased ADCC activity. Thus, even low levels of particular antibody subclasses significantly altered the effector profiles of humoral responses in these two vaccine trials, emphasizing the importance of the *functional quality* of antibodies. These findings encourage anti-HIV mAb engineering efforts to generate therapeutic IgG3s in addition to the typical IgG1 molecules that have dominated clinical mAb development thus far.

4.3.2 IgG Fc Engineering

Combinations of finer amino acid mutations and glycoengineering strategies to enhance Fc participation in ADCC (reviewed in [179, 221]), CDC [221], and ADCP [222] have generated an arsenal of Fc variants to incorporate into clinical mAbs. Studies have demonstrated that the *in vivo* efficacy of antibody therapeutics is determined by selective interactions of the Fc domain with activating and inhibitory Fc Receptors (FcRs) expressed on the surface of effector cells [216, 223]. Previous studies have implicated FcγRs in the protection afforded by HIV-specific antibodies [224-228] and demonstrated that Fc effector activity is necessary for the protective activity of anti-HIV mAbs in macaques [229] and humanized mice [230]. A recent study employing Fc domain engineering to selectively enhance or diminish engagement of activating FcγRs has demonstrated that the capacity to engage activating FcγRs predicts the *in vivo* efficacy of anti-HIV mAbs more accurately than *in vitro* neutralization activity: poorly neutralizing anti-HIV mAbs gained potent *in vivo* activity when expressed as IgG subclass variants capable of engaging activating FcγRs, and bNAbs exhibited reduced *in vivo* potency when their ability to engage activating FcγRs was compromised [214].

Protein engineering efforts to improve ADCC have roots in improving cancer mAb therapies and utilize a combination of rational engineering based on Fc/FcR co-crystal structures and directed evolution by yeast and bacterial display to generate Fc variants with enhanced FcγRIIIa binding [222, 231-233]. The most potent engineered variants (S239D-I332E) exhibit stronger binding to FcγRIIa and FcγRIIb in addition to FcγRIIIa, increase NK cell-mediated ADCC and macrophage-mediated ADCC [222], and potentially improved the *in vivo* efficacy of tumor immunotherapy anti-CD19 antibodies in monkeys [234]. A newly described approach to enhance ADCC combines amino acid mutations asymmetrically to generate Fc heterodimers with similar or superior potency in ADCC, increased stability in the CH2 domain compared to conventional Fc variants, and selectively improved d binding to activating FcγRIIa over inhibitory FcγRIIb [235].

Efforts to improve ADCC are motivated by studies demonstrating that the affinity ratio of immune complex binding to activating FcγRIIa vs. inhibitory FcγRIIb determines whether inflammatory or attenuated immune responses are set into motion [236]. Within the realm of HIV, antibodies from elite controllers and untreated progressors exhibit greater phagocytic activity, altered Fc domain glycosylation, and higher ratios of FcγRIIa: FcγRIIb binding compared to untreated progressors [115]. Identification of an Fc variant, G236A, with 15-fold enhanced FcγRIIa:FcγRIIb binding ratios, mediated enhanced ADCC by macrophages, and combinations with other mutations resulted in Fc variants with both high NK-cell-mediated ADCC and macrophage-mediated ADCC in the setting of cancer [237]. Such variants may be especially relevant for mAb targeting of viral tissue reservoirs where macrophages reside [133], in addition to viral suppression by phagocytosis of opsonized viral particles.

Lastly, strategies to improve CDC activity have similarly relied upon structural identification of C1q binding to the CH2 domain of IgG1 [238]. Whereas many engineered, CDC-enhanced variants exhibited decreases in ADCC activity [239, 240], the most potent variant to date (S276E-H268F-S234T + G236A-I332E) demonstrates 23-fold enhanced CDC activity and maintains ADCC activity similar to wild-type [240]. Alternative approaches to enhance CDC include 1) mixed IgG1-IgG3 Fc variants: one variant (1133) consisting of the CH1, hinge, and a portion of the COOH-terminal CH3 domain from IgG1 and the Fc from IgG3 enhanced both CDC and ADCC activity of antitumor antibodies against CD52 in a cynomolgus monkey model [241], 2) mixed IgG-IgA “cross-isotype” variants which additionally binds to FcαRI comparably to IgA and mediates greater CDC than IgG1 or IgA Abs [242], and 3) the creation of self-assembling hexameric IgG [243].

4.3.3 IgG Fc Glycoengineering

Within an IgG subclass, further variation in immune activation results from >30 different glycoforms possibly present on the Fc domain at Asn297 of the heavy chain [244] which can affect the conformation of the Fc and its ability to interact with FcγRs. Three sugar residues in particular dramatically alter antibody Fc binding to FcRs: 1) fucosylation of the mannose core decreases binding affinity to FcγRIIIA [245], 2) sialylation of terminal galactose groups allows for lectin receptor binding to initiate anti-inflammatory cascades [246], and 3) outer arm

galactosylation enhances binding to C1q and FcγRI [247-249], decreases binding to FcγRIIIA [228], and can implement anti-inflammatory responses through immune complex-mediated associations of FcγRIIB with dectin-1 [250]. Antibody glycosylation profiles of HIV infected individuals exhibiting spontaneous control and improved antiviral activity have demonstrated a global shift toward agalactosylated glycoforms and an even more dramatic shift of HIV-specific antibodies toward agalactosylated, afucosylated, and asialylated glycans [228].

Glycoengineering efforts to improve therapeutic antibodies are still relatively new but supported by studies demonstrating that preparations of antibodies from different expression cell lines bear distinct glycosylation profiles and different capacities to trigger effector functions (reviewed in [251]). Glycoengineering efforts to decrease fucosylation [245, 252] and/or sialylation [253] may be accomplished through antibody production in modified cell lines, including mammalian (CHO), insect, yeast, and plant cell lines [254, 255], or through the use of specific enzymes that allow rational remodeling of antibodies' Fc bound N-glycan structures [256, 257]. A non-fucosylated variant of HIV bNAbs b12 predictably increased FcγRIIIA binding and ADCC activity *in vitro* but unexpectedly did not enhance protection against viral challenge *in vivo* [258], possibly due to maximal ADCC activity already achieved by the wild type b12, or due to a greater relevance of other FcγR-mediated functions to *in vivo* protection. Because non-fucosylated antibodies have often led to higher efficacy in other settings, this study highlights the importance of understanding the *in vivo* contributions of a given bNAbs and its Ab effector functions to prevention, therapy, and cure to better direct Fc engineering efforts towards clinically relevant goals.

Combined protein and glyco-engineering efforts can improve effector functions [241, 245, 259, 260] but in some cases improve binding affinity to FcγRs without eliciting stronger effector function [258, 261, 262]. These examples suggest that affinity thresholds for effector function exist that may already be met by single protein- or glyco-engineered variants, so enhanced affinity does not improve activity [221]. However, combining the two strategies can expand the breadth of mAb binding to FcγRs and C1q to increase the range of effector mechanisms through

which therapeutic mAbs can recruit innate immunity [241, 260, 263], which may be particularly useful in combatting viral reservoirs in various tissue compartments, each with potentially different profiles of innate immune cell populations.

5. **Adding non-native functions to bNAbs**

Engineering anti-HIV mAb therapies offers the opportunity to move a step beyond naturally occurring bNAbs to add non-native functions for enhanced clinical utility in HIV prevention, therapy, and cure. Many such strategies exist and include conjugation of antiviral mAbs or mAb-based molecules to: 1) cholesterol to enhance potency through cholesterol-mediated interactions with the viral membrane [264], 2) small molecule inhibitors of viral entry to enhance neutralization potency [265], 3) CD4 domains to expose CD4-inducible epitopes on viral Env [266-268], and 4) recombinant immunotoxins to enhance cytotoxicity [269-271]. In this review, we focus on the recent development of various bispecific formats (Figure 3) to combat viral resistance and T-cell engaging molecules/cells to eradicate latent HIV reservoirs ([summarized in Table 3](#)).

5.1 **Bispecifics**

Encouraging results from studies combining mAbs to optimize neutralization potency and breadth and to limit viral resistance have inspired the development of bispecifics which allow targeting of two epitopes with a single therapeutic agent (reviewed in [138]). Furthermore, low surface densities of Env and the trimeric Env structure represent viral evasion strategies to prevent bivalent inter- or intra-spike binding of natural IgGs [272] which can be overcome by divalent (Fab)₂ molecules with linker lengths permitting bivalent intra-spike binding [273]: mono- and bi-specific (Fab)₂ molecules demonstrated linker length-dependent enhancement of neutralization potencies and, in some cases, neutralization of viral strains resistant to conventional IgGs [273]. Thus, bispecifics may benefit from avidity effects to enhance potency and prevent viral escape beyond that observed from combinations of mAbs.

A recent study applied the CrossMab heavy chain heterodimerization strategy [274] to generate four bispecific combinations of HIV bNAbs exhibiting high breadth (VRC07 & 10E8) and high potency (PGT121 & PG9-PG16-RSH) [275]. All four bispecifics neutralized 94-97% of viruses in a panel of 206 HIV-1 strains and generally demonstrated potencies intermediate to that of the two parental mAbs, with the exception of VRC07xPG9-16-RSH which was 6.9 and 2.2-fold more potent than VRC07 and PG9-16-RSH respectively against dual-sensitive viruses. The physical combinations of the two parental mAbs [were](#) more potent than equivalent

concentrations of the bispecific IgGs except for the VRC07xPG9-16-RSH combination, which demonstrates that some bispecifics may demonstrate enhanced clinical efficacy compared to combination mAb therapy. Similarly, CrossMab generated anti-HIV bispecifics targeting HIV-1 Env and cellular receptors, such as PRO140-10E8, have demonstrated exceptional breadth and potency of neutralization [276].

Alternative bispecific formats include two variable domains from each of two mAbs to form tetravalent molecules (Figure 3): 1) tetravalent dual variable domain Ig molecules (DVD-Igs), in which a second specificity VH&VL domain is linked directly to a full-length IgG, as was done to combine HY (an affinity matured b12) and 7B2 (anti-gp41) [277], and 2) Morrison-type bispecifics in which scFvs are linked via (G4S)_n linkers to either the C or N terminus of the LC or HC of an IgG, as was done to generate tetravalent anti-CCR5 bispecifics [168] and of PG9-/PG16-iMAb [167]. PG9-iMAb and PG16-iMAb DVD-Igs exhibited picomolar potency and were able to neutralize viruses resistant to both parental mAbs—the authors hypothesized that iMAb bound to CD4 and thereby concentrated PG9/16 at sites of viral entry to increase the potency of PG9/16 domains. Similarly, tetravalent anti-CCR5 bispecifics exhibited 18-57 fold potency compared to parental Abs, with one bispecific demonstrating activity against strains resistant to each parental Ab. Both of these examples demonstrate the synergistic effects resulting from tetravalent bispecific combinations but again were only observed for certain combinations of mAb specificities. On the other hand, HY-7B2 DVD-Igs simultaneously targeting gp120 CD4bs and gp41 epitopes generally performed only equally to the more effective parental Ab HY in neutralizing virus, although they did expectedly improve binding avidity and could more effectively deliver cytotoxic immunoconjugates than either parental Ab alone. Other bispecifics simultaneously targeting gp120 and gp41 have demonstrated enhanced neutralization activity compared to the parental Abs alone and are thought to hinder the function of Env by cross-linking gp120 and gp41 [278]. Although these two studies utilized different parental mAbs, they suggest that the format of DVD-Igs and bispecifics can be optimized to bind both epitopes on either the same or adjacent viral Env for enhanced neutralization activity.

5.2 Engaging T-cell responses

Two antibody-based strategies, bispecific T-cell engaging molecules and chimeric antigen receptor T cells, harness the power of cytotoxic T cells to eradicate latent HIV-infected cells. Combining humoral and cytotoxic responses in this way may be especially relevant for HIV given associations of high circulating levels of HIV-1-specific cytotoxic T-lymphocytes (CTLs) in long-term non-progressors [279, 280] and HIV-exposed seronegative subjects [281].

5.2.1 Bispecific T-cell engagers

Most recently, bispecifics with one specificity for T-cell engagement have shown significant promise in combatting viral reservoirs in “kick & kill” strategies. Bispecific T-cell engaging molecules have largely been designed as immunotherapy in the treatment of various cancers, and the first US FDA approved bispecific T-cell engaging antibody, blinatumomab, was recently approved in 2014 for use in refractory acute lymphoblastic leukemia, further demonstrating the clinical safety and efficacy of these engineered molecules [282]. In HIV-targeted “kick and kill” strategies, avid binding effects from both HIV Env- and T Cell CD3-specific arms of the bispecific to infected cells may allow for cross-linking of CD3 to trigger latent HIV-infected CD3⁺ T cells to reactivate and produce viral particles and concurrently display more viral Env on their surface. Alternatively, the addition of HIV latency reversing agents may provide or boost the “kick” to induce viral production. After induction of increased Env expression on the surface of latently infected cells, the anti-CD3 moiety of the bispecific may then cross-link CD3 on cytotoxic CD8⁺ T cells to induce cytolysis.

Historically, bispecific molecules to target HIV latent reservoir cells paired soluble versions of the CD4 receptor to anti-CD3 T cell engaging antibody fragments in a variety of formats [283-285]. Previous attempts to employ anti-HIV mAb specificities in bispecific formats with anti-CD3 demonstrated limited efficacy, likely due to limited breadth [284-286]. More recently, two groups have incorporated anti-HIV mAbs with broader recognition of diverse viral strains into bispecific T-cell engaging molecules: one group employed non-neutralizing mAbs recognizing CD4i (A32) and gp41 (7B2) epitopes [287], while another utilized bNAb VRC07 recognizing the CD4bs epitope [288]. As mentioned previously, non-neutralizing antibodies recognizing epitopes not normally present on viral surfaces may be helpful against latently infected cells, which often display monomeric gp140 or gp41 stumps. Both of these bispecific T-cell engaging molecules directed autologous T-cell-mediated cytolysis of primary HIV latently infected cells *in vitro* using patient PBMCs. Furthermore, one study demonstrated *in vivo* safety in ART-treated macaques and an absence of increased viral replication despite activation of latent cells in the presence of ART [288]. Additionally, a combination of T-cell engaging bispecifics targeting different viral Env epitopes demonstrated increased potency *in vitro* [287], and supports the incorporation of additional anti-HIV mAb specificities for potential use in combination therapy. These early promising results support future *in vivo* testing to determine the extent of bispecific permeation of tissue compartments where viral reservoir cells are likely to

be found, as well as whether physiologic effector:target cell ratios in reservoir compartments of HIV-infected individuals influences the effectiveness of bispecific T-cell engaging molecules.

5.2.2 Chimeric Antigen Receptors (CARs)

Clinically, CAR T cells are increasingly demonstrating success for the treatment of cancers [289-292] and have been proposed for use in combatting HIV viral reservoirs [293, 294]. Furthermore, the first clinical use of “off-the-shelf” CAR T-cells in an infant who was administered the therapy on a compassionate basis in the UK was recently reported to demonstrate safety and efficacy [295], and makes the universal applicability and potential of HIV-specific CAR T cell therapy more viable.

HIV Env-targeting extracellular domains are fused to T cell CD3 ζ cytoplasmic domains, which may contain additional costimulatory signal intracellular domains to activate T-cell cytotoxicity of Env-expressing cells. Env-targeting CAR T-cells thus far have utilized components from the CD4 receptor [296-298], gp41-specific mAb 98.6 [296], CD4bs mAb F105 [299], and, most recently, a CD4-17b bispecific (17b targets a CD4-inducible epitope) [300]. The CD4-based CAR, composed of the CD4 extracellular domain linked to the CD3 ζ signaling chain (termed CD4 ζ), is the most well-studied and has undergone clinical trials demonstrating durability of CAR expression and CAR T-cell proliferation in patients with predicted $t_{1/2}$ > 16 years [301]. Encouragingly, CAR T-cell trafficking to rectal tissue and decreased viral load within rectal tissues was observed in patients with acute viremia [302], but, ultimately, no significant antiviral effects were found on plasma viral levels in these individuals, nor in the more long-lived viral reservoirs of patients with chronic infection [303]. However, these studies used first-generation CARs containing only the CD3 ζ intracellular domain. In contrast, the recently described CD4-17b bispecific CAR represents a second generation CAR with the addition of costimulatory signaling domains from CD28 [300], and third-generation CARs with further incorporation of survival signals from 4-1BB (CD137) or OX40 (CD134) to enhance clinical efficacy, as demonstrated in various cancer settings [304], could similarly enhance HIV-specific CAR candidates. Furthermore, candidates from recently isolated, more potent bNAbs could be incorporated into CARs, either alone or in bispecific formats to combat resistance similar to mAb therapy. Interestingly, the authors of the CD4-17b bispecific CAR observed linker length-dependent effects on potency of viral suppression [300], which could further be tuned in bispecific CARs employing bNAbs targeting different epitopes. Similar to bispecific T-cell engagers, identification of [Env](#) epitopes [that are](#) most widely expressed and accessible on

reservoir cells will be critical to HIV CAR development, which may give new purpose to non-neutralizing antibodies that recognize monomeric gp140 or gp41 stumps.

Introducing CARs to human embryonic stem cells and induced pluripotent stem cells has also recently been described and demonstrated to produce NK cells with the ability to inhibit HIV replication in CD4⁺ T cells *in vitro*, although *in vivo* HIV suppression was not significantly different for engineered cells compared to unmodified cells [305]. However, similar to CD4ζ-CAR T cell studies, redesign as 2nd or 3rd generation CARs containing additional costimulatory domains or incorporation of bNAb extracellular domains could be beneficial. Interestingly, hematopoietic stem progenitor cells have previously been engineered to express bNAbs prior to directed differentiation into Ab-secreting plasmablasts with demonstrated protection *in vitro* and *in vivo* [306, 307]. Thus, combining both CAR-engineered NK-/T-cells and bNAb-secreting B cells/plasmablasts from stem progenitor cells offers a chance to simultaneously direct cytotoxic, humoral, and innate immune responses against HIV.

6. Engineering Delivery Strategies

Targeted and durable administration may further improve the clinical efficacy of bNAbs for protection, therapy, and cure of HIV infection. Delivery of bNAbs to mucosal surfaces may better prevent infection by sexual transmission, whereas systemic circulation may better ensure broad surveillance and targeting of infected cells and latent reservoirs. In all applications, longer-lived responses decrease the frequency of administrations required and decrease the burden placed on patients. In this review, we discuss strategies to deliver recombinant bNAb proteins and bNAb-encoding genes for endogenous production.

6.1 Protein Delivery

Preventing sexual transmission of HIV in high-risk populations such as that found in sub-Saharan Africa has been difficult for a variety of social, economic, and cultural factors. Women are particularly vulnerable and often unable to negotiate safer sex with their partners [308]. Thus, female-initiated pre-exposure prophylaxis methods are in great need, and thus far development efforts have largely focused on topical microbicides and oral ART. Both of these methods require daily or coitus-dependent application and have suffered from patient non-adherence in multiple clinical trials [309]. bNAb immunotherapies offer a longer-acting (months), coitus-independent alternative to current strategies. Intravenous or intramuscular injections of bNAbs could deliver months of protection at a time but would require clinic visits and may be less available to wider populations as a result. A recent NHP study administering simianized bNAb

VRC01 demonstrated durable protective antibody concentrations lasting for 108 days and protecting from viral challenge 52 days after the last dose, serving as a model for clinical dosing schedules of passively-delivered bNAbs: six antibody infusions (loading phase every 2 weeks followed by maintenance phase every 2 months) could provide complete protection for a year [310].

While topical formulations have demonstrated efficacy in humanized mice with direct application to the exposure site [123], microbicide trials suggest that the reliance on adherence may undercut the effectiveness of this delivery strategy. As an alternative, intravaginal rings (IVR) have previously been developed for sustained delivery of small molecule drugs to combat non-adherence [311]. A recent study has developed an IVR for controlled release of IgG antibodies and demonstrated sustained release for 14 days *in vitro* [312], although translation to the *in vivo* mucosal environment remains to be seen. As the realm of recombinant protein therapeutics expands, additional delivery strategies for controlled release of such “biopharmaceuticals” (reviewed in [313]), such as injectable polymer depot strategies, could additionally be applied to HIV bNAb pre-exposure prophylaxis.

6.2 Gene delivery (Plasmid, lentiviral, AAV)

An alternative delivery strategy to achieve durable plasma circulation is to induce long-term expression of monoclonal bNAbs via plasmid, lentiviral, or adeno-associated virus vector based gene delivery strategies (reviewed in [134]). *In vivo* electroporation of Fab-[encoding](#) plasmids has demonstrated rapid generation of functional VRC01 Fab molecules in mouse sera lasting for at least 7 days after injection [314]. While this system suffers from relatively short-lived expression, it may significantly streamline the developmental process required to deliver bNAbs compared to traditional recombinant production/purification schemes.

Gene delivery using viral vectors enables more durable expression. Lentiviral delivery of genes encoding HIV-specific antibodies to human hematopoietic stem/progenitor cells (HSPCs), with their capacities for unlimited regeneration and multilineage differentiation, has demonstrated successful neutralization activity *in vitro* [307] and *in vivo* protection from HIV challenge in humanized mouse models [196, 306]. However, *ex vivo* transduction of human HSPCs faces challenges in isolating HSPCs in sufficient numbers, matching donors to patients, and may be difficult to scale up for production as a broadly applicable therapeutic.

Recent gene delivery approaches have largely focused on recombinant AAV (rAAV) vector gene delivery to skeletal muscle where the rAAV delivered genome can form stable non-integrating circular episomes that persist in non-dividing muscle cells [315-317]. Over 100

clinical trials demonstrate safety of transduction [318] and the first gene therapy product clinically approved for use in humans uses rAAV vectors to treat lipoprotein lipase deficiency [319].

AAV delivery of HIV-/SIV-specific neutralizing mAbs (reviewed in [320]) as full length-antibodies [321, 322] and immunoadhesin molecules (scFv+(G4S)₃+Fc) [320, 323] have demonstrated high expression levels with serum concentrations of delivered transgene proteins of up to 1000 μg/mL [322, 324] and significant durability of expression—one study found sustained serum concentrations of 20 μg/mL immunoadhesins for the past four years in macaques [320]. Significantly, 6 out of 9 macaques receiving an AAV-delivered SIV-specific immunoadhesin were completely protected from IV challenge with 40 macaque infectious doses of SIVmac316 [320, 323], with the lack of protection in the remaining three thought to result from significant immune responses to the immunoadhesin observed before viral challenge [323]. Similar protection from viral challenge has been observed from AAV delivered human bNAb transgenes in a dose-dependent manner in humanized mice [120, 310, 325].

AAV-delivered bNabs also protect from mucosal HIV transmission: AAV vector delivery of VRC01 family bNAbs protected the majority of humanized mice from 15 consecutive weekly vaginal challenges with the JR-CSF molecular clone of HIV and from 21 weekly vaginal exposures to a transmitted founder strain of HIV (REJO.c) [325]. Another study delivering a simian version of CD4bs bNAb VRC07 demonstrated protection against SHIV infection in monkeys 5.5 weeks after treatment and circulating antibody levels up to 66 μg/mL with the addition of immune suppression drugs (cyclosporine) [326]. AAV-delivery of an immunoadhesin scFv-Fc based on HIV bNAb PG9 has recently entered phase I clinical trials (www.clinicaltrials.gov NCT01937455).

Furthermore, AAV-delivery of a *non-neutralizing* anti-SIV mAb, 5L7, in full IgG1 format to rhesus monkeys resulted in endogenously produced anti-SIV mAb concentrations ranging from 1-270 μg/mL, with lower concentrations reflecting host antibody responses to the delivered mAb observed in 9 out of 12 cases, as well as lower peak and setpoint viral load and delay in time to peak viral load from time of exposure [327]. One monkey with the highest serum concentrations of 5L7 completely resisted six successive SIVmac239 IV challenges and exhibited extraordinarily high serum ADCC activity on a per μg basis greater than the equivalent concentration of recombinantly produced 5L7. The authors postulated that this extraordinarily high ADCC activity likely results from differences in post-translational modifications of the endogenously produced 5L7, further supporting glycoengineering efforts to tune Fc effector function and an additional potential advantage of AAV-delivered bNAb gene therapy over passive transfer of protein.

One major potential limitation of AAV therapy arises from preexisting immunity [328, 329] since ~80% of people are seropositive for either the AAV1 or AAV2 capsid [52]. Thus, efforts to identify capsids from other species [55] or to engineer entirely new capsids [56,57] may be important for the clinical utility of AAV therapy. Potential immunogenicity and polyreactivity of delivered bNAbs may also require bNAb engineering strategies, although no anti-antibody responses have been observed in passive administration of anti-HIV mAbs in human trials thus far [14, 330].

7. Conclusion

Anti-HIV antibodies offer significant promise for prophylactic protection from establishment of infection, therapeutic control of viremia after infection, and even functional cure of infection through combinations of virus neutralization, antibody-mediated effector functions, and added functionalities from conjugation to drugs, proteins, or whole cells. Recent technological advances have enabled rapid isolation of bNAbs with increased potency and breadth that have demonstrated exciting successes in several preclinical animal models and inspired the return of anti-HIV mAbs to human clinical trials. Sequence and structural information from isolated bNAbs and viral escape mutations inform Fv-engineering strategies to increase Ab potency and breadth, counter viral resistance, and decrease polyreactivity and solubility issues. Deeper understanding of extra-neutralizing roles for antibodies redefine the clinical utility of non-neutralizing antibodies and greatly inform engineering efforts to improve Fc regions of clinical anti-HIV mAbs to increase antibody half-life, improve mucosal immunity, and enhance recruitment and activation of innate immunity. Addition of small molecules, multiple antibody specificities, soluble receptors, toxins, or T-cell-engaging scFv's/cells may prevent viral escape or even provide a functional cure through targeting of latent HIV reservoirs. Finally, various delivery strategies to localize recombinant mAbs at sites of infection or to deliver mAb-encoding genes for long-lived therapy significantly increase the clinical utility and feasibility of anti-HIV mAbs for prevention, therapy, and functional cure of HIV infection. While further studies to evaluate viral resistance, immunogenicity, and adverse reactions to several of the strategies discussed in this review remain to be conducted, the current arsenal of tools with which to enhance anti-HIV antibodies for clinical use is expansive and offers opportunities to exercise ingenuity and innovation to outpace HIV infection.

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<u>Goal</u>	<u>Strategy</u>	<u>Model Ab</u>	<u>Enhancement</u>	<u>Reference(s)</u>
<u>↑ Potency and/or breadth of neutralization</u>	<u>Directed evolution</u>	<u>b12</u>	<u>↑ Affinity ~400x & ↑ breadth</u>	<u>139,140</u>
		<u>m9</u>	<u>↑ Breadth through sequential antigen panning</u>	<u>141</u>
	<u>Rational mutations</u>	<u>PG9 N100(F)Y</u>	<u>Stabilize CDR-H3 in active conformation</u>	<u>142</u>
		<u>NIH45-46 G54W, VRC07-523</u>	<u>Improve Hydrophobic interactions</u>	<u>65, 143</u>
		<u>45-46m2</u>	<u>Leverage glycan contacts</u>	<u>144</u>
		<u>45-46m2, 45-46m7, 45-46m25, and 45-46m28</u>	<u>Avoid steric clashes between Ab & Ag escape variants</u>	<u>144</u>
		<u>10-1074&PGT121, PG9-PG16-RSH, 4E10&10E8</u>	<u>Combine CDRs of bNAbs targeting similar epitopes</u>	<u>60, 69, 145</u>
		<u>CD4-Ig</u>	<u>Replace Fv with extracellular domain of CD4</u>	<u>147-150, 153, 154</u>
<u>Restrict viral escape</u>	<u>Rational mutations</u>	<u>45-46m7, 45-46m25, and 45-46m28</u>	<u>Bias antigen escape towards detrimental mutations</u>	<u>144</u>
	<u>Combining mAbs</u>	-	<u>Combine mAbs with complementary resistance patterns</u>	<u>156-158</u>
	<u>Target host cellular receptors</u>	<u>Ibalizumab (iMAb)</u>	<u>Target CD4 receptor</u>	<u>159-162</u>
		<u>PRO140</u>	<u>Target CCR5 coreceptor</u>	<u>159, 163-166</u>
		<u>bNAb + iMab / PRO140</u>	<u>Bispecifics combining bNAbs with iMab or PRO140</u>	<u>167, 168</u>
<u>↓ Polyreactivity/ aggregation propensity</u>	<u>Rational mutations</u>	<u>NIH45-46 G54W, 10E8</u>	<u>Determine acceptable mutations based on clonal relatives</u>	<u>65, 143</u>

Table 1. Summary of HIV bNAb Fv engineering strategies.

<u>Goal</u>	<u>Strategy</u>	<u>Modification</u>	<u>Enhancement</u>	<u>Reference(s) for modification</u>	<u>HIV-specific reference(s)</u>
<u>↑ Half-life</u>	<u>↑ FcRn binding</u>	<u>CH2-CH3 domain</u>	<u>longer serum half-life, increased localization to mucosal epithelial surfaces</u>	<u>184-186, 190</u>	<u>181</u>
<u>↑ Mucosal immunity</u>	<u>Reformatting as IgA</u>	<u>Isotype switching</u>	<u>enhanced mucosal localization & effector function</u>	<u>192-193</u>	<u>192, 193</u>
	<u>↑ binding to FcαR</u>	<u>"Cross-isotype" IgG-IgA: IgGA</u>	<u>↑ macrophage-mediated ADCP & Neutrophil-mediated ADCC</u>	<u>242</u>	
<u>↑ Effector function</u>	<u>IgG Subclass Switching</u>	<u>IgG Fc switching</u>	<u>skewing towards particular effector functions</u>	<u>215-216</u>	<u>214</u>
	<u>↑ Binding to activating FcRs by protein engineering</u>	<u>↑ FcγRIIIa binding</u>	<u>↑ ADCC</u>	<u>222, 231-234</u>	<u>214, 229-230</u>
		<u>↑ FcγRIIIa:FcγRIIb</u>	<u>↑ ADCP & ADCC</u>	<u>235, 237</u>	
		<u>↑ C1q binding</u>	<u>↑ CDC</u>	<u>239, 240</u>	
		<u>Mixed IgG1-IgG3 Fc</u>	<u>↑ CDC & ADCC</u>	<u>241</u>	
		<u>"Cross-isotype" IgG-IgA: IgGA</u>	<u>↑ ADCC, ADCP, ADCDC</u>	<u>242</u>	
		<u>Self-assembling hexameric IgG</u>	<u>↑ CDC</u>	<u>243</u>	
	<u>↑ Binding to activating FcγRs by glycoengineering N297</u>	<u>↓ Fucosylation</u>	<u>↑ FcγRIIIa binding --> ↑ ADCC</u>	<u>245, 252</u>	<u>258</u>
	<u>Mixed protein & glyco-engineering</u>	<u>↓ Sialylation</u>	<u>↓ lectin receptor-mediated anti-inflammatory cascades --> ↑ ADCC</u>	<u>253</u>	
		<u>Mixed IgG1-IgG3 Fc + afucosylated</u>	<u>↑ CDC & ADCC</u>	<u>241</u>	
		<u>Aglycosylated (bacterial expressed) + ↑ FcγRI binding, no FcγRIIIa/IIb binding</u>	<u>↑ ADCC</u>	<u>260</u>	
		<u>Engineering the protein-carbohydrate interface</u>	<u>↓ ADCC</u>	<u>263</u>	<u>-</u>

Table 2. Summary of HIV bNAbs Fc engineering strategies. Note the listed references for modifications represent a subset of applicable Fc engineering strategies.

<u>Goal</u>	<u>Strategy</u>	<u>Example</u>	<u>Type</u>	<u>Reference(s)</u>
<u>Restrict viral escape</u>	<u>Bispecific molecules</u>	<u>3BNC60, b12, 10-1074, PG16</u>	<u>(Fab)₂ molecules: both homo- and hetero-epitopic, permit intra-spike crosslinking</u>	<u>273</u>
		<u>VRC07 & 10E8; PGT121 & PG9-PG16-RSH; bNAb & iMab/PRO140</u>	<u>CrossMab heterodimerization bispecifics, potential synergism</u>	<u>275, 276</u>
		<u>HY + 7B2</u>	<u>Tetravalent dual variable domain Ig molecules (DVD-Igs), potential synergism</u>	<u>277</u>
		<u>two epitopes on CCR5; PG9/PG16-iMab</u>	<u>Tetravalent Morrison-type bispecifics, potential synergism</u>	<u>167, 168</u>
<u>Engage T-cell responses: targeting viral reservoirs</u>	<u>"Kick and kill" bispecifics</u>	<u>A32 (CD4i) / 7B2 (gp41) & anti-CD3</u>	<u>Dual-Affinity Re-Targeting Proteins (DARTs)</u>	<u>287</u>
		<u>VRC07 & anti-CD3</u>	<u>Bispecific immunomodulatory protein</u>	<u>288</u>
	<u>Chimeric antigen receptors (CAR)</u>	<u>CD4EC Domain; 98.6; F105; CD4-17b bispecific</u>	<u>CAR T-cells</u>	<u>296-298, 196, 299, 300</u>
		<u>CD4EC Domain</u>	<u>CAR embryonic stem cells</u>	<u>305</u>

Table 3. Summary of HIV bNAb engineering strategies to introduce non-native functions.

Figure Legends

Figure 1. Structure of the HIV Envelope glycoprotein based on the crystal structure of BG505 SOSIP.664 (PDB ID: 4TVP). A] Ribbon representation of HIV gp140 protomer and trimers composed of gp120 (green) and gp41 (purple) subunits. B] Surface structure of HIV Env trimer colored by degree of conservation in the 2014 HIV Sequence Compendium from the LANL database [331]. C] Surface epitope domains colored by contact residues and residues $\leq 4\text{\AA}$ from contact residues: V1V2V3 loop/trimer apex (red); V3 glycan-N332 supersite (orange); CD4 binding site (green); gp120-gp41 interface (blue); gp41 membrane-proximal external region (purple). Common bNAbs against each epitope domain are listed. Glycans are not shown, but importantly mask a significant portion of the viral Env surface. Images were generated using the UCSF Chimera package.[332]

Figure 2. Antibodies and antibody-based molecules. A] Cartoon representations of antibody isotypes IgG, monomeric IgA (mIgA), dimeric IgA (dIgA), and secretory IgA (sIgA) are depicted and color coded as follows: heavy chain (blue); light chain (orange); disulfide bonds (black lines); J chain (red); secretory component (purple line). Constant heavy domains 1-3 (CH1-CH3), constant light domain (CL), variable heavy (VH), variable light (VL), antigen-binding fragment (Fab), and crystallizable fragment (Fc) domains are also annotated. B] Antibody-based molecules include the single-chain variable fragment (scFv), antigen-binding fragment (Fab), and scFv-Fc immunoadhesin molecules. Linkers are represented by blue lines. C] CD4-Ig molecules replace scFv/Fab regions with CD4 extracellular domains 1 & 2 (CD4 D1D2 in green). eCD4-Ig additionally includes a C-terminal CCR5 mimetic peptide (CCR5mim1).

Figure 3. Bispecific antibody formats. Cartoon representations of Fab- and scFv- based bispecifics (A), divalent Fc-containing bispecifics (B), and tetravalent bispecific antibody formats (C) are depicted and color-coded by parental mAbs: mAb 1 HC (blue) and LC (orange); mAb 2 HC (purple) and LC (red).

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Fig. 1

Fig. 2

Fig. 3

A

Bispecific scFv
(Bis-scFv)

Dual Affinity Re-
Targeting Protein
(DART)

Bispecific (Fab)₂

scFv-Fab

B

CrossMab
Bispecific IgG

C

Bispecific scFv-Fc
immunoadhesin

Morrison-type Bispecifics

DVD-Ig

Graphical Abstract