

REVIEW

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Adeno-associated virus gene delivery of broadly neutralizing antibodies as prevention and therapy against HIV-1

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Abstract

Vectored gene delivery of HIV-1 broadly neutralizing antibodies (bNAbs) using recombinant adeno-associated virus (rAAV) is a promising alternative to conventional vaccines for preventing new HIV-1 infections and for therapeutically suppressing established HIV-1 infections. Passive infusion of single bNAbs has already shown promise in initial clinical trials to temporarily decrease HIV-1 load in viremic patients, and to delay viral rebound from latent reservoirs in suppressed patients during analytical treatment interruptions of antiretroviral therapy. Long-term, continuous, systemic expression of such bNAbs could be achieved with a single injection of rAAV encoding antibody genes into muscle tissue, which would bypass the challenges of eliciting such bNAbs through traditional vaccination in naïve patients, and of life-long repeated passive transfers of such biologics for therapy. rAAV delivery of single bNAbs has already demonstrated protection from repeated HIV-1 vaginal challenge in humanized mouse models, and phase I clinical trials of this approach are underway. Selection of which individual, or combination of, bNAbs to deliver to counter pre-existing resistance and the rise of escape mutations in the virus remains a challenge, and such choices may differ depending on use of this technology for prevention versus therapy.

Keywords: Vectored delivery, Antibody gene transfer, AAV, HIV-1, bNAb, Clinical trials, Animal models

Background

HIV-1 remains a significant contributor to the global burden of disease. In 2016, 1.8 million individuals were newly infected with HIV-1, and more than 36 million individuals were living with HIV-1, of whom only 44% were virally suppressed with antiretroviral therapy (ART) [1]. The need for daily dosing of ARTs remains a challenge for their effective use for both viral suppression as well as pre-exposure prophylaxis of HIV-1. Whether due to lack of drug access, stigma, inability, or drug-drug interactions, failure to maintain drug pressure in the body can result in breakthrough infection or drug-resistant viral rebound. Long-term, continuous, systemic expression of anti-HIV-1 antibodies by a single administration of recombinant adeno-associated viruses (rAAV) may be an alternative to ARTs.

This review will summarize advances in using recombinant AAV (rAAV) for gene transfer, and describe broadly neutralizing antibodies (bNAbs) against HIV-1 and the results of recently completed clinical trials that passively transfer these bNAbs into individuals living with HIV-1. It also describes recent progress of vectored delivery of bNAbs for long-lasting expression in humanized mouse models, macaque models, and in ongoing clinical trials, and concludes with the challenges faced in deciding which bNAbs to deliver.

Main text

Recombinant adeno-associated viruses (rAAV) for gene transfer

AAVs have long been contemplated as attractive vectors for use in gene transfer [2]. AAV is a replication-defective 20–25 nm *Parvoviridae* virus consisting of a non-enveloped, icosahedral protein shell (capsid) surrounding one copy of a linear single-stranded DNA genome. Initially

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found in 1965 as a contaminant of adenovirus preparations [3], AAV can only replicate within cells in the presence of helper functions provided by viruses such as adenovirus or herpesvirus. The 4.7 kb AAV genome encodes for *rep* and *cap* in between two palindromic 145 bp inverted terminal repeats (ITRs). These ITRs self-anneal into T-shaped hairpin structures [4]. *rep* is translated into four non-structural proteins for packaging and replication and *cap* into three structural capsid proteins that protect the genome and modulate cell binding and trafficking. In addition, a recently discovered alternative open-reading frame in *cap* encodes for assembly-activating protein, which is necessary for capsid assembly in certain AAV serotypes [5]. Thirteen serotypes of AAV (named AAV1-13) have been discovered to date, and these serotypes differ in tissue tropisms, transduction efficiencies, and expression levels dependent on their viral capsid sequence [6]. Screening in humans and non-human primates and ancestral sequence reconstruction have identified numerous additional infectious capsids that are variants of the 13 representative serotypes [7–9].

The ITRs are the only sequence elements required *in cis* for packing of the genome into the capsid and for replication. Thus, recombinant AAV (rAAV) vectors used for gene transfer need only consist of an expression cassette encoding a promoter and transgene placed between the ITRs, in lieu of *rep* and *cap*. Helper functions of *rep* and *cap* are supplied *in trans* via a separate plasmid, co-transfected during production, and thus no viral genes are encoded by rAAV. The serotype choice for *cap* provided *in trans* dictates the identity of the capsid shell of the recombinant vector and thus which tissues are preferentially infected by rAAV. Given the importance of *cap* in modulating tissue tropism and possibly immunogenicity [10, 11], numerous efforts are underway to engineer *cap* for greater specificity and desirable activities [12].

AAVs have no apparent pathogenicity, as they are not known to be associated with any human disease [13]. Natural AAV infection occurring without helper virus functions can enter a latent phase and integrate site-specifically into the AAVS1 site on the 19th chromosome in humans, in a process that requires proteins encoded by *rep* [14–16]. Because rAAV vectors do not encode *rep*, their genomes persist as extrachromosomal episomal concatemers that rarely integrate into the chromosome [17–19]. Despite the episomal nature of rAAV, a single intramuscular injection of rAAV has been shown to maintain transgene expression for a number of years in a variety of animal models including humans [20–23], in one case enabling detection of rAAV transgene expression in a patient over 10 years after administration [24].

There are several general considerations to using rAAV as a gene transfer vector. First, rAAV has a limited transgene carrying capacity. AAV has a genome of 4.7 kb, and rAAVs that are produced with transgenes of more than approximately 5 kb result in substantially reduced transduction efficiencies [25]. Second, transgene expression upon transduction of target tissues with single-stranded rAAV is not immediate, as the cell must first synthesize the second strand using the single-stranded DNA genomic template [26, 27]. Lastly, pre-existing immunity of individuals to AAV from natural exposure may limit the efficiency of transduction. Global seroprevalences of different AAV serotypes range from 30 to 60% [28, 29]. Even if transduction is able to occur, adaptive immune responses can severely limit transgene expression. In early gene therapy trials using AAV2 to deliver factor IX to patients with hemophilia B, factor IX expression was limited to only several months, likely due to transduced cells presenting AAV capsid peptides, which reactivated memory T cells targeting those transduced cells [30]. Subsequent trials using AAV8 were successful in stable expression of factor IX when they excluded patients with detectable anti-AAV antibodies and used the lowest dose of rAAV8 that still provided therapeutic benefit [23, 31]. Efforts to discover rare and ancestral AAV capsids and to create new capsids for which humans do not yet have an immune response are underway [9]. Since a patient who receives rAAV will likely develop immunity against the capsid upon injection, subsequently giving the same patient another rAAV with the same capsid serotype is unlikely to result in additional transgene expression.

Only two rAAV gene therapy products have been licensed to date, but many more are in clinical trials [32]. Glybera (alipogene tiparvovec) for lipoprotein lipase deficiency was the first gene therapy product licensed in Europe in 2012, in which the human lipoprotein lipase gene in an AAV1 capsid is administered via intramuscular injection. Luxturna (voretigene neparvovec) for inherited retinal dystrophy was the first gene therapy product approved by the FDA in 2017, in which the RPE65 gene in an AAV2 vector is injected subretinally to treat blindness [33, 34]. Affordability and patient accessibility of gene therapy products remain to be determined. Priced at 1 million dollars per treatment, Glybera was withdrawn from the market by its manufacturer after 5 years [35]. Due to difficulty of convincing national reimbursers to pay for the treatment, it was only used in one patient. Luxturna has been similarly priced at \$425,000 per eye [36]. To increase its acceptability, its manufacturer is seeking reimbursement only with positive outcomes. The prices of future gene therapy products will depend on the commercial outcomes of these initial products and further maturation and widespread adoption of these technologies.

Anti-HIV-1 broadly neutralizing antibodies (bNAbs)

Gene transfer of anti-HIV-1 broadly neutralizing antibodies (bNAbs) with rAAV may be an effective method to prevent and suppress HIV-1 infection. Approximately half of chronically infected HIV-1 individuals naturally develop sera capable of neutralizing half of the diversity of HIV-1 at low to moderate titers [37]. However, only a small proportion of individuals develop bNAbs of great potency and breadth that cross-clade neutralize diverse HIV-1 strains, by binding to conserved regions of the HIV-1 envelope spike. These rare bNAbs are heavily somatically hypermutated from years of coevolution with the virus [38]. Several properties of HIV-1 envelope impede the development of such antibodies. First, a single HIV-1 virion displays only ~14 envelope spikes on its surface [39]. Such low-density surface protein limits the potential for avidity effects and thus may result in less BCR cross-linking for B cell activation. Second, the envelope surface is covered by shifting glycosylation sites and flexible variable loops that sterically hinder access to conserved epitopes buried deep inside the protein, and thus antibodies against HIV-1 are more likely to be strain-specific than broad [40, 41].

Nevertheless, improvements in antibody discovery techniques have resulted in the identification of new bNAbs each year [42]. Antibodies appear to bind to several preferential target regions on the HIV-1 envelope: the V1/V2 site at the trimer apex, the N332 glycan super-site near the V3 loop, the CD4 binding site, the gp120–gp41 interface, and the membrane-proximal external region (MPER) [43]. The CD4 binding site is of particular interest as it is well conserved due to the need for HIV-1 to bind to its primary receptor for infection. bNAbs that target the CD4 binding site include b12 [44], VRC01 [45, 46], 3BNC117 [47], N6 [48], and N49P7 [49]. These latter antibodies possess great breadth and potency, as N49P7 neutralized 86% of a 117 multi-clade pseudovirus panel at an $IC_{50} < 1 \mu\text{g/ml}$ [49], and N6 neutralized 96% of another 181 multi-clade pseudovirus panel at an $IC_{50} < 1 \mu\text{g/ml}$ [48].

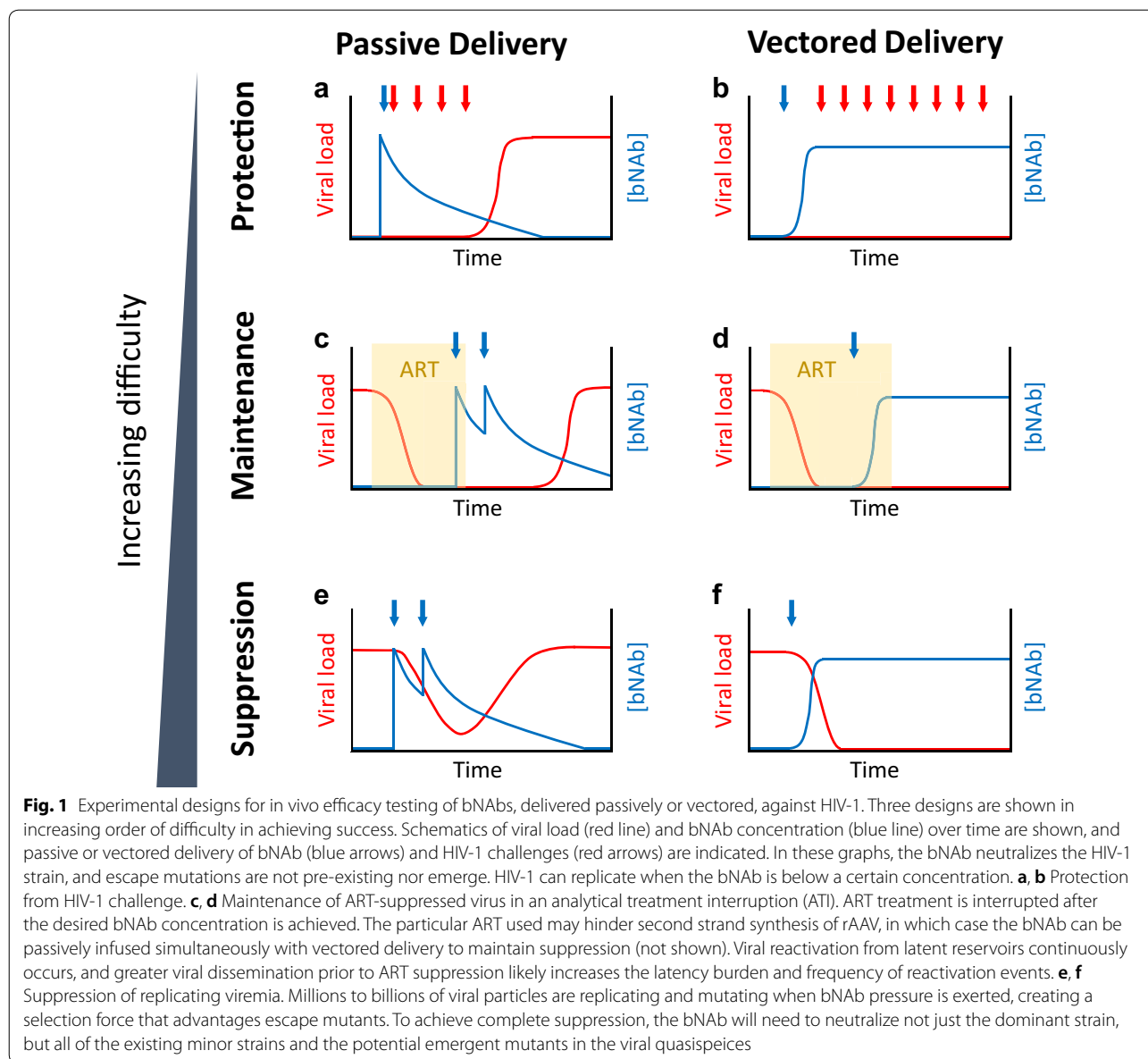
However, eliciting bNAbs in individuals through vaccination is likely to be difficult as a consequence of the extensive somatic hypermutation and unusually long sequence complementarity determining regions observed in many bNAb lineages. Thus, novel sequential administration of different immunogens may be needed to elicit bNAbs in patients [50]. Multiple immunogen design strategies have emerged to first stimulate bNAb germline precursors and then drive affinity maturation against bNAb target epitopes [51]. In lineage-based immunogen design, immunogens mimic natural viral evolution found in a patient who develops a bNAb, starting with the founder strain [52]. In germline-targeting immunogen

design, the first immunogen seeks to engage bNAb germline precursors. For instance, eOD-GT8 is a multivalent nanoparticle, presenting a structure-based design of gp120 outer domain molecule selected through iterative random mutagenesis and yeast cell surface display [53]. Priming with this immunogen followed by more native Env-like boosts in a VRC01 germline knock-in model resulted in antibodies of intermediate VRC01 maturity [54]. Results of these approaches are promising, but guiding such maturation in diverse patient populations may be difficult due to allelic diversity at immunoglobulin loci. Given the likely difficulty of eliciting bNAbs via traditional vaccines, alternative approaches using existing bNAbs either through passive transfer or gene transfer are being explored.

Passive transfer of bNAbs in clinical trials

Given the challenges of eliciting highly somatically mutated bNAbs in naïve individuals, direct administration of mature bNAbs for prevention or therapy is currently being tested in humans. Experimental designs for these clinical trials are shown in Fig. 1. Six phase I or IIa trials of passive infusion of single bNAbs (VRC01 [55, 56], 3BNC117 [57, 58], and 10-1074 [59]) into HIV-1 infected individuals have been published to date (Table 1), with many more underway or planned [60]. These studies use one of two therapy trial protocols. The first consists of administration of antibody into viremic individuals and observing the decline in viral load and time until viral rebound (Fig. 1e). The second is an analytic treatment interruption (ATI), where ART-suppressed HIV-infected individuals are given multiple sequential antibody infusions and taken off ART shortly after the first infusion (Fig. 1c). The delay in latent viral rebound is then observed. Overall, these studies have shown that the examined bNAbs have a therapeutic effect and exert selection pressure on the virus. The degree of suppression varied across antibodies and patients, depending on antibody potency and the presence of pre-existing resistance mutations in a patient. Interestingly, despite theoretical concerns over the degree of somatic hypermutation these antibodies exhibit, these bNAbs have not been found to be particularly immunogenic in humans, as anti-drug antibody (ADA) responses have not been observed in these trials. Across all of these trials, bNAb half-life was consistently shorter in HIV-1 infected individuals than in uninfected individuals, perhaps due to increased clearance of antibody-antigen immune complexes.

The first clinical trials in HIV-1 infected individuals examined CD4 binding site bNAbs. In a phase I trial, Caskey et al. [57] administered a single infusion of 3BNC117 to eight HIV-1 infected, viremic individuals, which significantly reduced mean viremia from baseline



for 4 weeks by up to 1.5 log₁₀ copies/ml. A subsequent 3BNC117 ATI phase IIa trial gave multiple infusions in 13 HIV-1 infected individuals [58]. Individuals were pre-screened for PMBC viral outgrowth cultures with 3BNC117 sensitivity (IC₅₀ ≤ 2.0 μg/ml). Viral rebound was significantly delayed by a mean of 6.7 weeks in individuals with 2 infusions, or by a mean of 9.9 weeks in individuals with 4 infusions, compared to historical controls of 2.6 weeks. 3BNC117 levels at viral rebound ranged from 6 to 168 μg/ml, and these values correlated with the IC₈₀ of recrudescent viruses.

Two findings from these 3BNC117 trials suggested that 3BNC117 imposed a high barrier to viral escape. First, in

a majority of participants of the second ATI trial (8/13), the recrudescent viruses were at least threefold more resistant by IC₈₀. In 5 out of these 8, the rebounded virus appeared to have emerged from a single resistant provirus. In contrast, virus rebound after standard analytical treatment interruption without additional treatment is typically polyclonal, as multiple latent viruses are reactivated [61]. The restriction of recrudescent viruses suggests that 3BNC117 was preventing the rise of most latent clones. The virus that rebounded would have pre-existed at low frequencies such as not to have decreased bulk pre-infusion virus neutralizability. The first trial similarly found that recrudescent viruses with a reduction in

Table 1 Clinical trials of bNABs in HIV-1 infected individuals with published results

Clinical trial	Trial design	Delivery	bNAB	Highest dose given (mg/kg)	HIV-1 infected individuals given highest dose	Dosing schedule	bNAB sensitivity prescreening	Viral response	References
NCT02018510	Suppression	Passive	3BNC117	30	8 viremic individuals	One dose	Some	Viral load was reduced by mean of 1.5 log ₁₀ copies/ml (range of 0.8–2.5), and significant for 28 days	[57]
NCT02446847	Maintenance	Passive	3BNC117	30	13 suppressed individuals	2 doses 3 weeks apart, or 4 doses 2 weeks apart; ART discontinued 2 days after first dose	All	2 infusions delayed rebound by mean of 6.7 weeks (range of 5–9) after ATI, and 4 infusions by mean of 9.9 weeks (range 3–19)	[58]
NCT01950325 (VRC 601)	Suppression	Passive	VRC01	40	8 viremic individuals	One dose	None	Amongst responders (6/8), viral load was reduced by 1.1–1.8 log ₁₀ copies/ml, and significant for 21 days	[55]
NCT02463227 (ACTG A5340)	Maintenance	Passive	VRC01	40	14 suppressed individuals	Dose 1 week before and 2 and 5 weeks after ART discontinuation	None	Rebound was delayed by 4 weeks (IQR 3–5) after ATI	[56]
NCT02471326 (NIH 15-0140)	Maintenance	Passive	VRC01	40	10 suppressed individuals	Dose 3 days before, 2 weeks after, and each subsequent month after ART discontinuation	None	Rebound was delayed by 5.6 weeks (IQR 4.1–5.6) after ATI	[56]
NCT02511990	Suppression	Passive	10-1074	30	13 viremic individuals	One dose	None	Amongst responders (11/13), viral load was reduced by mean of 1.5 log ₁₀ copies/ml (range 0.9–2.1), and significant for 27 days	[59]

3BNC117 sensitivity tended to cluster in low-diversity lineages. Second, in the ATI trial, of the four individuals who were suppressed until antibody concentration fell below 20 µg/ml, three appeared to not have gained 3BNC117 resistance. Thus, a resistant mutant failed to emerge in the presence of the antibody, demonstrating antibody potency.

Three phase I trials of VRC01 passive infusion found that VRC01 could similarly suppress HIV-1, although suppression seemed to be less than that of 3BNC117. In Lynch et al. [55], in the 8 viremic patients given the highest dose, mean viral load was lower than baseline for 3 weeks. Individuals appeared to follow one of three patterns. Two individuals with mostly pre-existing resistant viruses did not respond, and two individuals with baseline viral loads less than 1000 copies/ml were briefly undetectable and then remained below baseline for at least 6 weeks. The last four individuals had sensitive viruses with a 14 to 59-fold reduction in viral load, but their virus started rebounding after 10 days. Except in the first two individuals with already completely resistant viruses, rebounded viruses had decreased sensitivity to VRC01 after infusion. These rebounded viruses were polyclonal except in one individual, where a pre-existing resistant minor lineage expanded to dominate the population.

In the two other VRC01 ATI trials, viral rebound was delayed by 4 or 5.6 weeks [56]. In a majority of individuals, the rebounded virus was polyclonal. Individuals with detected pre-existing resistant viruses had earlier viral rebounds, and individuals with pre-existing resistance throughout their viral diversity were more likely to have a polyclonal rebound. VRC01 resistance increased in most participants after infusion.

The 10-1074 trial tested a bNAb with an epitope outside of the CD4 binding site [59]. In this trial, 11 out of the 13 individuals who received the highest dose responded with a mean decrease of 1.5 log₁₀ copies/ml, and the decrease was significant for almost four weeks. The two other individuals harbored pre-existing resistance and did not respond. Mean serum concentration was 77 µg/ml at rebound.

Across all VRC01 and 3BNC117 trials, mutations were observed occurring in or near the V5 loop, the D loop, and the CD4 binding site, epitopes common to CD4 binding site antibodies. In contrast, escape mutations in the 10-1074 trial were concentrated to the well-defined potential N-linked glycosylation N332 sequon (PNGS) and a ³²⁴G(D/N)IR³²⁷ motif. Resistance to 10-1074 was highly polyclonal in individual patients, suggesting that there were multiple ways the virus could escape antibody neutralization without greatly sacrificing viral infectivity and replicative fitness. Interestingly, the authors also

found that baseline codon composition at these sites influenced the observed escaped mutations. Specifically, one individual, who in pre-treatment harbored a TCT serine codon instead of the more common AGT serine codon at the S334 PNGS, exhibited single point mutations at that codon to a different set of amino acids than other individuals after 10-1074 infusion. Lastly, in 5 out of 6 individuals sequenced after 10-1074 was no longer detectable, the N332 sequon and ³²⁴G(D/N)IR³²⁷ motif re-emerged, suggesting these escape mutations have sufficient *in vivo* fitness cost to necessitate reversion when antibody levels diminished.

Overall, these bNAbs are at least transiently efficacious in the therapeutic setting of suppressing viremia or preventing the emergence of latent viruses. In the case of 3BNC117 and VRC01, in patients where rebound virus emerged with reduced sensitivity to the infused bNAb, the rebound strains were often nearly identical and clustered together in low-diversity lineages in phylogenetic trees, distinct from the pre-existing quasispecies. This suggests that the bNAb bottlenecked the rebound virus—one or only a few strains escaped antibody pressure and subsequently expanded. These strains may have either been pre-existing at low-frequencies, or they represent chance emergence of resistant mutants during antibody therapy. In contrast, the rebound virus from 10-1074 was consistently polyclonal, suggesting that the barrier to escape may be lower for this bNAb. Whether this is because the 10-1074 epitope faces less selection pressure to remain as conserved as the CD4 binding site is unclear.

These clinical trials also found evidence that bNAbs have additional advantages over ARTs against HIV-1. In particular, 3BNC117 was found to have *in vivo* functionality beyond neutralization. 3BNC117 improved the anti-HIV-1 neutralization activity of autologous antibody responses and also increased clearance of infected cells through Fcγ receptor engagement [62, 63]. Further investigation of how these antibodies may engage additional innate immune functions such as antibody-dependent cellular cytotoxicity and phagocytosis *in vivo* is needed [64, 65]. This is particularly important in the context of HIV-1 cure as antibodies may target cells within the latent viral reservoir that have been reactivated to produce virus [66].

In contrast to clinical trials for therapy, clinical trials for prevention are harder to conduct, as many more patients need to be repeatedly reinfused to detect treatment significance. The ongoing Antibody Mediated Prevention (AMP) study (HVTN 703/HPTN 081, <https://clinicaltrials.gov/ct2/show/NCT02568215> and HVTN 704/HPTN 085, <https://clinicaltrials.gov/ct2/show/NCT02716675>) seeks to passively infuse VRC01 into thousands of trial participants every other month over the course of 10

infusions (Fig. 1a). Results from these trials are eagerly anticipated, as they are poised to be the first to show that bNAbs can actually prevent HIV infection in humans. Regardless of the outcomes of these trials, implementing continuous passive infusion globally, which requires repeated hospital visits from patients and cold chain transport, is infeasible. Steady-state antibody concentrations from passive transfer may also decline below prophylactic levels if the infusion schedule is delayed. Thus, vectored antibody delivery represents an attractive alternative for sustained bNAb production as a means of prevention. Sustained antibody levels, potentially achievable with gene transfer, may also result in long-term viral suppression, as suggested in the few 3BNC117 patients in whom viral rebound occurred only after antibody concentration greatly diminished and without increased resistance.

Efficacy of vectored delivery of bNAbs in animal models

Gene delivery of bNAbs may result in the sustained, systemic expression of such antibodies with as little as one rAAV intramuscular injection, in contrast to passive transfer (Fig. 1). In this approach, antibodies are endogenously produced in muscle cells, targeted for export with secretion peptides, and passively circulated around the body. Table 2 summarizes the studies of vectored delivery of bNAbs covered in this review, and Table 3 lists considerations in using different animal models.

Lewis et al. [67] first demonstrated sustained expression of a bNAb in mice in 2002. They delivered b12, a CD4 binding site bNAb, using an rAAV2 vector encoding both CMV and EF1- α promoters separately expressing the heavy and light chain genes. Injection into Rag1-immunodeficient mice resulted in peak serum levels of 4–9 $\mu\text{g/ml}$ after 12 weeks, and the extracted serum was biologically active when measured by *in vitro* neutralization assays against HIV-1.

Johnson et al. [68] in 2009 subsequently delivered anti-SIV immunoadhesins via AAV in macaques. These immunoadhesins were based on anti-SIV Fabs obtained from PCR amplification of bone marrow cells of SIV-infected macaques and selected using phage display [69]. The variable light and heavy chains of these Fabs were joined by a linker to make a single chain variable fragment (scFv), which was then fused to a rhesus IgG2 Fc fragment. The authors constructed two such immunoadhesins, 4L6 and 5L7, as well as N4 that contained domains 1 and 2 of rhesus CD4. rAAVs encoding each of these constructs were intramuscularly injected into three macaques using an AAV1 capsid. N4 was constructed as a single-stranded genome, and 4L6 and 5L7 as self-complementary genomes, where two halves of an inverted repeat genome fold into a double-stranded DNA

upon transduction, thereby bypassing the rate-limiting second-strand synthesis step. After 4 weeks, 4L6 or 5L7 immunoadhesin levels were 40–190 $\mu\text{g/ml}$, except for one macaque, in which 5L7 expression was eliminated due to the development of anti-5L7 antibodies. N4 levels were lower at 3–10 $\mu\text{g/ml}$. When these macaques were challenged intravenously with SIVmac316 a month after transduction, six out of nine animals were protected, but three were infected. Upon investigation, these three infected macaques had developed endogenous immunogenic responses against the immunoadhesins prior to challenge, thereby limiting the effectiveness of prevention.

In 2005, Fang et al. [70] first demonstrated the long-lasting delivery of full-length antibodies at therapeutic levels using single-stranded rAAV vectors. They achieved this by expressing a single open reading frame encoding the antibody heavy and light chains, linked by a 24-amino acid 2A self-processing sequence derived from picornavirus. Separation of these chains occurs between the last two residues of the 2A sequence through a ribosomal skip mechanism which prevents the formation of the peptide bond during translation [71]. A 4-amino acid furin cleavage sequence was added after the heavy chain and before the 2A sequence, which resulted in the removal of the residual 2A peptide in the Golgi. The single 2A amino acid at the N-terminus of the light chain is located prior to the signal peptide, and thus was not present in the mature antibody. Using this system, the authors demonstrated that injection of an rAAV8 vector carrying a VEGFR2-neutralizing antibody gene into mice resulted in >1 mg/ml expression of the antibody for over 4 months with *in vivo* therapeutic efficacy. In a follow-up study, Fang et al. [72] optimized the furin cleavage site to achieve more complete and uniform cleavage.

Our lab used these developments to demonstrate that full-length bNAbs identical to those found in humans could be continuously produced at therapeutic levels via AAV gene transfer and that such vectored immunoprophylaxis (VIP) can prevent intravenous HIV transmission [73]. In addition to using codon-optimized 2A and furin sequences in the expression cassette, we also developed a muscle-optimized promoter (CASI), made from combining a CMV enhancer, a chicken β -actin promoter, and a ubiquitin enhancer embedded within a synthetic intron. A woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) was included downstream of the antibody transgene to increase expression. For the vector, rAAV8 was used as it efficiently transduces non-dividing, post-mitotic muscle tissues, which have limited turnover, and human seroprevalence against AAV8 is lower than against AAV1 or AAV2 [74]. In addition, unlike AAV2, AAV8 does not activate capsid-specific T

Table 2 Evaluation of vectored delivery of bNAbs against HIV-1 in animal models

Experimental design	Model	Delivery	Antibody	Highest rAAV genome copies given	Challenge virus	Challenge route	Week of 1st challenge post vectored delivery	[Antibody] in serum at challenge	Viremia in experimental arm	Viremia in control arm	References
Expression	Rag1 KO mice	rAAV2	b12	5×10^{11}	HIV-1 IIIIB	N/A (in vitro neutralization with serum)	20	2–6 µg/ml	1/6 not neutralize	No control	[67]
Protection	Macaques	rAAV1	4L6, 5L7, or N4 immunoadhesins	2×10^{13}	SIVmac316	Intravenous	4	0–190 µg/ml (1/9 developed ADA)	3/9 after 1 challenge	6/6 after 1 challenge	[68]
Protection	HuPMBC-NSG mice	rAAV8	b12 or VRC01	1×10^{11}	HIV NL4-3	Intravenous	4	198–313 µg/ml	0/8 after 1 challenge	8/8 after 1 challenge	[73]
Protection	BLT mice	rAAV8	VRC01	1×10^{11}	HIV JR-CSF	Intravaginal	4	45–151 µg/ml	2/10 after 15 challenges	9/9, after mean 4.25 challenges	[77]
Protection	BLT mice	rAAV8	VRC07-G54W	1×10^{11}	HIV REJO.c	Intravaginal	4	56–118 µg/ml	0/13 after 21 challenges	12/12, after mean 7.45 challenges	[77]
Maintenance	Hu-CD34-NSG mice	rAAV2	10-1074	2.5×10^{11}	HIV YU-2-NL4-3	N/A	N/A	~200 µg/ml	1/7 rebounded	No control	[80]
Protection	Macaques	rAAV1	IgG1 versions of 4L6 or 5L7	1.6×10^{13}	SIVmac239	Intravenous	14 or 44	0–270 µg/ml (9/12 developed ADA)	11/12 after 6 challenges	6/6 after 6 challenges	[106]
Protection	Macaques, with cyclosporine administration (CsA) for 4 weeks	rAAV8	Simianized VRC07	1×10^{13}	SHIV-BalP4	Intrarectal	5.5	0–39 µg/ml (1/6 developed ADA during CsA, 2/5 after CsA)	2/6 after 1 challenge	5/5 after 1 challenge	[107]
Protection	Macaques	rAAV1/2	Rhesus eCD4-Ig with rhesus tyrosine-protein sulfotransferase 2	2.5×10^{13}	SHIV-AD8	Intravenous	8	17–77 µg/ml	0/4 after 6 challenges	4/4 after 6 challenges	[108]

Table 3 Considerations in model choice for evaluating vectored delivery of bNAbs against HIV-1

Characteristics	Hu-PBMC-NSG mice	Hu-CD34-NSG mice	Bone marrow-liver-thymus (BLT) mice	Macaques
Construction	Injection of expanded human PBMCs into NSG mice	Injection of fetal hu-CD34+ hematopoietic stem cells into newborn irradiated NSG mice	Injection of fetal hu-CD34+ hematopoietic stem cells into newborn irradiated NSG mice, along with surgical implantation of autologous thymus and liver tissue	Not needed
Antibody immunogenicity	(+) No strong anti-drug antibody response, permitting sustained antibody expression	(+) No strong anti-drug antibody response, permitting sustained antibody expression	(+) No strong anti-drug antibody response, permitting sustained antibody expression	(-) Strong anti-drug antibody responses, which is not fully resolved with antibody simianization
Immune functionality	(-) Activated human T-cell engraftment, but largely lacking other lineages, no hematopoietic regenerative source, no HLA-restriction, and no primary immune response	(-) Multi-lineage hematopoiesis with functional human T cell compartment that is mouse H2-restricted, but inconsistent humoral and Fc receptor effector responses	(+/-) Multi-lineage hematopoiesis with functional human T cell compartment that is HLA-restricted, but inconsistent humoral and Fc receptor effector responses	(+/-) Functional immune system, but is simian
Model longevity	(-) Up to 8 weeks due to GvHD	(+) Up to a year	(+) Up to a year	(+) Many years
HIV replication	(+) Supports HIV replication	(+) Supports HIV replication	(+) Supports HIV replication	(-) Does not support HIV replication (have to use either SIV or SHIV)
Challenge routes	(-) Intravenous only	(-) Intravenous only	(+) Intravenous, Intravaginal, Intrarectal	(+) Intravenous, Intravaginal, Intrarectal
Genetic homogeneity	(+) Genetic homogeneity within cohort with same graft, increasing reproducibility	(+) Genetic homogeneity within cohort with same graft, increasing reproducibility	(+) Genetic homogeneity within cohort with same graft, increasing reproducibility	(+/-) Genetic diversity, which can complicate analysis with small numbers
Similarity to human physiology and size	(-) Less	(-) Less	(-) Less	(+) More
Costs	(+) Lowest cost	(+) Low cost	(+/-) Moderate cost, but requires surgery	(-) High cost

cells, due to lack of heparin binding which likely leads to uptake by dendritic cells [75], and may induce immune tolerance [10, 76]. In a first experiment, rAAV8-b12 was transduced into huPMBC-NSG humanized mice. Antibody levels were sustained at levels greater than 100 µg/ml a month after transduction. Transduced mice were fully protected from a challenge dose of NL4-3 HIV-1 that was 100-fold higher than needed to infect 7 out of 8 control mice. In a second dose-response experiment, the minimum amounts of rAAV-b12 or rAAV-VRC01 to fully protect mice from NL4-3 HIV-1 infection were found to be 1.25×10^{10} genome copies in both cases, corresponding to a mean concentration of 34 µg/ml for b12 and 8 µg/ml for VRC01.

Subsequently, our lab demonstrated that VIP can also protect humanized mice from low-dose repetitive intravaginal challenge [77]. To better model mucosal transmission of HIV-1, we used a more advanced bone marrow-liver-thymus (BLT) humanized mouse model, as described later in this review. In a first experiment, humanized mice transduced with rAAV8-VRC01 were challenged weekly with JR-CSF, a clade B, R5-tropic virus, starting a month after transduction. Upon transduction, VRC01 was detected at 100 µg/ml in the serum and a minimum of 100 ng/ml in the cervicovaginal lavage fluid, which represented an underestimate of the actual mucosal concentration as antibody was diluted by the vaginal wash procedure. Control mice were infected after a mean of 4.25 exposures, whereas only two of ten transduced mice were infected after 13 and 15 exposures. In a second experiment, VRC07-G54W was delivered via rAAV8 to humanized mice a month before beginning weekly challenges with a transmitted founder clade B, R5-tropic virus, REJO.c. VRC07 was created by pairing the original VRC01 light chain with a newly discovered heavy chain from the VRC01 patient [78], and the G54W mutation increased the antibody potency via mimicry of Phe43 in CD4 [79]. In this experiment, control mice were infected after a mean of 7.45 exposures, whereas none of the mice given VRC07-G54W antibody were infected after 21 exposures. These works demonstrated the protective effect of bNAbs delivered via rAAV against intravaginal HIV-1 challenge in a humanized mouse model.

Other labs have shown that rAAV-delivered bNAbs can also be used for therapeutic purposes. Horwitz et al. [80] in 2013 demonstrated that suppression of HIV-1 can be maintained with rAAV gene transfer of bNAbs in the NSG-CD34+ humanized mouse model. Because they found that ART interfered with AAV transduction, they first suppressed the virus with ART, then passively infused a bNAb while withdrawing ART, and subsequently maintained suppression with rAAV delivery of the same bNAb. They found that rAAV2-10-1074

sustained antibody concentrations of approximately 200 µg/ml and maintained suppression of YU-2-NL4-3 HIV-1 in 6 out of 7 mice. Future work could explore the use of other ART combinations to eliminate the need for a passive infusion bridge.

These studies demonstrate that rAAV delivery can sustain expression of anti-HIV-1 bNAbs in humanized mouse models. Similar VIP approaches have also been shown to protect mouse models against other infectious diseases, such as influenza [81], malaria [82], HCV [83], and Ebola [84].

Evaluating vectored delivery in humanized mouse models

The natural immune response to HIV-1 is reflected to different degrees amongst the various humanized mouse models. The simplest mouse model infuses adult human T-cells derived from PBMCs into immunodeficient mice [85]. These mice support HIV-1 infection and ongoing viral replication, but the graft quickly depletes after several weeks as there is no regenerative source of T-cells. Furthermore, these mice develop graft-versus-host disease (GvHD) within 6–8 weeks as the graft is not tolerant of the foreign environment [73]. Another model involves the transplantation of human CD34+ stem cells into newborn immunodeficient mice [80, 86, 87]. This allows for the development of a regenerating T-cell compartment and improved longevity as a consequence of T-cell tolerance, which may be a consequence of human T-cell progenitors being educated in mouse thymus tissue. However, the lack of human thymus results in an immune system that is unable to recognize peptides presented in the context of human HLA molecules and thus a largely incompetent adaptive immune response to infection. The most complete humanized mouse model is the bone marrow-liver-thymus (BLT) mouse model, in which newborn immunodeficient mice are surgically implanted with tissue fragments from human fetal liver and thymus, followed by intravenous injection of purified autologous fetal human CD34+ hematopoietic stem cells derived from the remainder of unimplanted liver [88–90]. The T cell compartment of the BLT mice reconstitutes over several months and T cells mature in the transplanted human thymus and are largely tolerant to mouse antigens. The BLT mouse can model multiple aspects of HIV-1 infection, such as prevention, viral evolution in response to T-cell pressure, mucosal transmission, CTL responses, and viral latency [77, 91–94].

Humanized mouse models have the benefit of genetic homogeneity when engrafted with tissue from the same donor. Isogenic cohorts can be as large as tens to over one hundred and fifty mice, allowing for the observation of chance behaviors in response to HIV-1 infection. These humanized mouse models are also significantly

less expensive than macaque models. Importantly, natural human bNAbs with specificity against HIV can be tested in humanized mice without eliciting strong ADA responses that confound experiments in other systems.

However, the existing BLT mouse model does not yet faithfully replicate all aspects of a fully-functional human immune system. Our lab and others have observed inconsistent humoral responses against viral proteins in BLT mice during HIV-1 infection. This could be due to previous observations of disorganized germinal centers, and defects in antigen presentation. In addition, the BLT model does not fully capture the pharmacokinetics and pharmacodynamics of human bNAbs given the murine origin of the neonatal Fc receptor (FcRn) recycling receptors [95]. Fc-mediated behaviors beyond neutralization, such as antibody-dependent cell-mediated cytotoxicity (ADCC) may not be well supported in this model due to a paucity of natural killer cells. Furthermore, complement-dependent cytotoxicity (CDC) is not supported in this model due to genetic defects in the underlying NSG mouse strain complement cascade [96].

Evaluating vectored delivery of bNAbs in macaques

Evaluating the effectiveness of rAAV gene transfer of anti-HIV-1 bNAbs in nonhuman primates has proven to be challenging, due to a lack of naturally-existing, effective antibodies against SIV and the propensity of macaques to develop strong immunogenic responses against human bNAbs. However, macaques are better models of human physiology, have fully functional immune systems that are analogous to that of humans, and are of more comparable size. Importantly, HIV-1 does not replicate in macaques and thus a closely related virus, SIVmac, is used to model HIV-1 infection and the corresponding AIDS-like symptoms that develop [97]. However, while functionally similar, SIV and HIV-1 share only about 50% sequence homology, and SIV encodes an additional accessory protein (Vpx), not found in HIV-1, that induces degradation of host restriction factor SAMHD1 [98, 99].

Given that human bNAbs do not recognize SIVmac, a chimeric virus (SHIV) is often used for antibody-mediated protection experiments in which the SIVmac envelope is replaced with an HIV-1 envelope [100]. However, this chimeric virus is not fully adapted and may be of lower fitness than natural SIV strains, as unlike HIV-1 in humans or in BLT humanized mice, some SHIV strains are occasionally controlled in untreated macaques [101, 102]. Interestingly, not all HIV-1 envelopes can be made into functional SHIV, although substantial progress has been made in doing so [103]. In addition, unlike mice, macaques are not inbred, and genetic diversity in a cohort may lead to disparate immunological responses to infection that can complicate analysis of divergent

behavior within a group. Most importantly, as reviewed in this section, human bNAbs appear to elicit significant immunogenic responses in macaques [104, 105], and these responses are not seen when passively transferring bNAbs in humans.

Fuchs et al. [106] in 2015 constructed full-length rhesus IgG1 counterparts of the 4L6 or 5L7 immunoadhesins used in Johnson et al. [68] and delivered them via rAAV1 to macaques. Although antibody concentrations reached 20–300 µg/ml, endogenous ADA responses were detected within a month in three of the six macaques given 5L7, and in all six macaques given 4L6. These ADA responses limited continual expression of the antibody, as antibody concentrations fell below 10 µg/ml in macaques that developed ADA responses. The authors then repeatedly challenged the macaques with SIVmac239, a strain more difficult to neutralize than SIVmac319 used by Johnson et al. Although neither gene transfer appeared to be more effective at preventing infection than the negative control, 5L7 delayed time to peak viral load, and lowered peak and set viral loads. It was later found that the variable regions of these antibodies were immunogenic, which contributed to their xenogeneic elimination [105].

Other works have sought to simianize bNAbs to reduce cross-species immunogenicity with mixed results. Saunders et al. [104] in 2015 found that a single infusion of human VRC01 resulted in detectable anti-VRC01 plasma IgG eight weeks after infusion, but not for simianized VRC01. They subsequently infused simianized simVRC01 or simVRC01-LS four times over 8 months into eight macaques, and found that the bNAb could persist for more than 2 or 3.5 months, respectively, after the last infusion. However, in two of the eight macaques, ADA response was nonetheless detected against the simianized antibody. The six other macaques were intrarectally challenged with SHIV-BaLP4 2 months after last the passive infusion, of which five were protected.

In a parallel study, Saunders et al. [107] evaluated whether administering immunosuppressant cyclosporine before rAAV injection reduced immunogenicity against bNAbs. The authors first delivered simianized VRC07 via rAAV8 to macaques. Serum concentrations peaked at 2.5–7.7 µg/ml at weeks 2–4, and substantial ADA response was detected. The same constructs given at a lower dose to immunodeficient mice resulted in levels greater than 100 µg/ml. In a second study, six macaques were given cyclosporine starting 9 days prior to, and until 4 weeks after, rAAV8-simVRC07 administration. Peak mean concentration of simVRC07 was 38 µg/ml. Three of six macaques retained simVRC07 expression for 16 weeks without developing ADA, whereas the others exhibited ADA, including one that completely eliminated simVRC07 expression. Macaques were intrarectally

challenged with SHIV-BaLP4 5.5 weeks after transduction, and the two of the six macaques with the lowest simVRC07 concentrations became infected. Overall, ADA response was inversely correlated with simVRC07 concentration, and transient immunosuppression did increase transgene expression.

Since bNAbs, even simianized, were still immunogenic after long-term expression in macaques, Gardner et al. [108] in 2015 took an alternative approach and delivered rh-eCD4-Ig, which is rhesus CD4-Ig fused at its carboxyl terminus to a 15-amino acid CCR5-mimetic sulfopeptide, in an rAAV1/2 vector into macaques. Rhesus tyrosine-protein sulfotransferase 2 was also co-administered in a second rAAV at a 1:4 ratio to increase rh-CD4-Ig sulfation, as this is required for its neutralizing activity. rh-eCD4-Ig was expressed at 17-77 $\mu\text{g}/\text{ml}$ for more than 40 weeks and protected four macaques from four increasingly stringent challenges with SHIV-AD8. Less anti-transgene response was detected against rh-eCD4-Ig than against simianized 3BNC117, NIH45-46, 10-1074, PGT121, and no antibody response against the sulfopeptide was detected.

In summary, there are substantial challenges associated with evaluating the long-term expression of bNAbs in nonhuman primates, through both repeated passive infusions and rAAV gene delivery. Although simianization, in which human variable regions are engrafted onto a rhesus antibody, reduces bNAb immunogenicity in passive transfer studies, it does not fully eliminate ADA responses as those variable regions are descended from the human germline and not from their simian counterparts. Thus, it may be inherently difficult to evaluate the anti-HIV-1 efficacy of sustained human bNAb expression in macaques with confounding xenogenic responses. Immunosuppressants such as cyclosporine reduce but do not completely eliminate the immunogenicity of bNAbs [107]. Future studies aiming to deliver fully simian antibodies against SIV or HIV, cloned in a manner analogous to methods used to isolate human bNAbs, may more accurately predict the potential for translation of this approach in humans.

Vectored delivery of bNAbs in clinical trials

There are currently only two bNAb AAV gene transfer studies. The first trial (IAVI A003/CHOP HVDDT 001, <https://clinicaltrials.gov/ct2/show/NCT01937455>) is a phase I trial in 21 healthy males without HIV-1 or HIV-2 infection and uses an rAAV1 vector encoding PG9 heavy and light chain under two separate promoters. Either 4×10^{12} , 4×10^{13} , 8×10^{13} , or 1.2×10^{14} viral genomes regardless of weight or placebo were given in a single intramuscular administration to participants without evidence of pre-existing anti-AAV1 antibodies.

These participants were then followed for a year, with the option of enrolling into a follow-up study. This trial was completed in February 2018, but results have not yet been reported in the literature. The second trial (VRC 603, <https://clinicaltrials.gov/ct2/show/NCT03374202>) is a phase I trial in an estimated 25 adults living with suppressed HIV-1 infection and uses an rAAV8 vector encoding a CASI-promoter driven VRC07 transgene in a nearly identical configuration to those used in our previously published studies [73, 77]. Participants in VRC603 must have controlled viremia, have been on stable ART for at least 3 months, and not have evidence of pre-existing anti-AAV8 antibodies. Either 5×10^{10} , 5×10^{11} , or 2.5×10^{12} viral genomes per kg will be given in a single intramuscular administration to the upper arm or thigh, with a study goal of achieving 50 $\mu\text{g}/\text{ml}$ 4 weeks post-injection and a set point of 5 $\mu\text{g}/\text{ml}$ 12 weeks post-injection. Participants will be followed intensely for a year, and then every 6 months for another 4 years. This trial has an estimated primary completion date of March 2019. Longer-term follow up of patients in these trials, past these primary study completion dates, is desirable to evaluate the duration of sustained bNAb expression and the potential immunogenic responses from chronic bNAb exposure.

Selection of bNAbs for vectored delivery

Since the characterization of PG9 in 2009 [109], over 90 bNAbs have been described, exhibiting a wide range of breadth and potency as determined by neutralization assays on large global panels of HIV-1 isolates [42, 110]. Given that most infections are initiated by a single transmitted founder virus [111, 112], low in vivo bNAb concentrations that reduce the probability of the establishment of infection may be sufficient to provide a benefit in the context of prevention. As more potent antibodies are discovered, the prophylactic dose of bNAb necessary to yield protective concentrations is likely to be reduced.

In contrast, suppressing actively replicating virus with bNAb is more challenging given the millions to billions of virions that must be neutralized. In addition, instead of a single viral genotype, the bNAb is faced with neutralizing a quasispecies of closely related HIV-1 strains [113]. Since the virus mutates as it replicates, the quasispecies may harbor a variant that evades antibody neutralization, thereby allowing for escape. Such variants that escape bNAb pressure may face a replicative fitness penalty, particularly if conserved sites such as those involved in interacting with CD4 are mutated. It may be useful to consider the fitness costs of escaping each bNAb, and use antibodies that impose a high escape cost when optimizing

bNAb delivery, particularly in a therapeutic setting [114, 115].

Another feature specific to rAAV delivery is that bNAb expression may take several weeks to achieve steady state levels [73, 77], as second strand synthesis of the rAAV genome is necessary for expression to occur [26, 27]. From the viral perspective, this steadily rising concentration of antibody represents a gradually increasing selective force which may more readily select for escape mutants. Use of a stronger promoter or of a more efficient AAV serotype may result in faster expression. Alternatively, co-administration of both an adenovirus vector and an AAV vector can result in immediate and sustained antibody expression, as previously shown for a monoclonal antibody against anthrax [116].

Similar to existing HAART regimens, which employ a combination of antiretroviral drugs to control HIV-1, the use of antibody combinations to suppress HIV-1 has been long proposed and may be necessary, particularly in the context of therapy [117, 118]. Klein et al. [119] repeatedly passively infused either a tri-mix or penta-mix of bNAbs into YU-2-NL4-3 HIV-1 infected humanized mice. The tri-mix (which neutralized >98% of clades with IC_{80} of 0.121 $\mu\text{g/ml}$) led to complete suppression in 3 of 12 mice, and the penta-mix (which neutralized >98% of clades with IC_{80} of 0.046 $\mu\text{g/ml}$) led to complete suppression in 11 of 13 mice. This and other work suggests that antibodies which bind to different epitopes and with very low IC_{50} across diverse HIV-1 strains should be chosen [119–121]. Bispecific monoclonal antibodies, where each arm of the antibody binds to a different epitope, may also have greater breadth and potency than each constituent antibody alone or mixed [122, 123]. rAAV delivery of bispecifics may require two separate rAAVs injected into the same site, due to the carrying capacity of the vector, but this has not yet been reported in the literature. Another approach is to study viral mutants that arise after administration of a single bNAb and then design variants of that bNAb that neutralize those mutants. In particular, Diskin et al. [124] rationally designed NIH45-46 variants by increasing the buried surface area of the antibody with escape variants and avoiding steric clashes. However, when NIH45-46 and its variants were passively infused as a combination into infected humanized mice, mutants escaped in a previously unseen path by shifting an N-linked glycosylation site by three residues, highlighting the magnitude of the challenge of designing antibody combinations to suppress actively evolving viremia.

Conclusion

Phase I and IIa trials of passive transfer of bNAbs have thus far demonstrated the safety of bNAbs in humans and shown that bNAbs can both transiently lower viral

loads and delay viral rebound. In some patients, viral rebound happened only after the bNAb concentration fell to low concentrations and occurred without escape mutations, suggesting that continuous bNAb expression might result in sustained suppression. Given the difficulty of eliciting bNAbs through sequential vaccination and the complexity of life-long passive transfer of bNAbs, using a single intramuscular administration of rAAV to attain continuous, systemic, long-term expression of bNAbs is an exciting possibility.

rAAVs have a favorable safety profile and can stably express transgenes in humans for many years. However, pre-existing immunity in individuals against AAV due to natural exposure may limit successful vectored delivery. To avoid this, immunosuppressants may be temporarily administered, or novel AAV capsids with little cross-reactivity to circulating AAVs may be used [125]. In humanized mouse models, vectored delivery resulted in the long-term expression of bNAbs, protected against intravenous and intravaginal HIV-1 challenges, and maintained suppression of previously ART-suppressed HIV-1. However, pre-existing and emergent viral resistance to bNAbs may limit their effectiveness in patients. Whereas bNAbs may be able to neutralize a few slightly resistant virions in the context of prevention, using bNAbs to suppress replicating HIV-1 may require additional innovations to prevent the evolution and selection of viral mutants. Prevention studies in macaques with AAV-delivered simianized bNAbs elicited strong anti-bNAb responses, although it seems likely that the immunogenicity of the human antibody variable regions in macaques complicates this model. Most importantly, Phase I clinical trials of vectored delivery of bNAbs are currently underway and should provide critically important information to determine the feasibility of this approach. Irrespective of their outcome, we believe that whatever challenges may be encountered will ultimately be overcome and that vectored delivery of broadly neutralizing antibodies will become an important new approach towards ending the HIV-1 epidemic.

Abbreviations

AAV: adeno-associated virus; ADA: anti-drug antibody; ART: antiretroviral therapy; ATI: analytical treatment interruption; BCR: B-cell receptor; BLT: bone marrow-liver-thymus; bNAb: broadly neutralizing antibody; HIV: human immunodeficiency virus; ITR: inverted terminal repeat; PNGS: potential N-linked glycosylation sequon; rAAV: recombinant adeno-associated virus; SIV: simian immunodeficiency virus; VIP: vectored immunoprophylaxis.

Authors' contributions

AL and ABB conceived of the review together. AL wrote the review. AL and ABB edited the review together. Both authors read and approved the final manuscript.

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