HHS Public Access

Author manuscript

Immunol Rev. Author manuscript; available in PMC 2018 January 01.

Published in final edited form as:

Immunol Rev. 2017 January ; 275(1): 324–333. doi:10.1111/imr.12478.

Antibody Gene Transfer with Adeno-Associated Viral Vectors as a Method for HIV Prevention

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Summary

Broadly-neutralizing antibodies (bNAbs) against human immunodeficiency virus (HIV) show great promise in HIV prevention as they are capable of potently neutralizing a considerable breadth of genetically diverse strains. Passive transfer of monoclonal bNAb proteins can confer protection in animal models of HIV infection at modest concentrations, inspiring efforts to develop an HIV vaccine capable of eliciting bNAb responses. However, these antibodies demonstrate high degrees of somatic mutation and other unique characteristics that may hinder the ability of conventional approaches to consistently and effectively produce bNAb analogues. As an alternative strategy, we and others have proposed vector-mediated gene transfer to generate long-term, systemic production of bNAbs in the absence of immunization. Herein, we review the use of adeno-associated virus (AAV) vectors for delivery of HIV bNAbs and antibody-like proteins and summarize both the advantages and disadvantages of this strategy as a method for HIV prevention.

Keywords

Adeno-associated virus; antibody gene transfer; broadly neutralizing antibodies; HIV; immunoprophylaxis

Introduction

The field of HIV vaccine research has been invigorated by the discovery of broadly-neutralizing antibodies (bNAbs). While natural infection predominantly elicits non-neutralizing or strain-specific antibodies, 10–30% of HIV-infected individuals generate bNAbs approximately 2–4 years after infection (1–3). These unusual antibodies are capable of neutralizing most circulating strains of HIV, making them promising candidates for HIV prevention. In fact, numerous *in vivo* studies in non-human primate (NHP) models have clearly demonstrated their potential to confer protection in models of HIV transmission. For example, passive transfer of bNAb proteins directed against various epitopes of the HIV

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Conflicts of Interest

There are no conflicts of interest.

envelope glycoprotein (Env) has effectively protected animals from intravenous challenge of SHIV, a chimeric SIV/HIV virus (4). Similar studies have extended these results to show that numerous bNAbs of varying epitope specificity can protect animals from both intravenous and mucosal SHIV transmission (5–8). Passive transfer of 2G12, an HIV bNAb recognizing a glycan-dependent Env epitope, led to reduced viral load in newborn macaques highlighting the potential of bNAbs to impact mother-to-child transmission (9).

In addition to animal models, several phase I clinical trials have demonstrated the efficacy of bNAbs against HIV infection. A single intravenous infusion of VRC01, a bNAb directed against the CD4 binding site (CD4bs) of HIV Env, reduced viral load by 1.1–1.8 $\log_{10}$ in 6 of 8 viremic subjects (10). Those who did not respond were found to have predominantly VRC01-resistant virus at the onset of the study. Similarly, a single intravenous infusion of 3BNC117, another CD4bs-directed bNAb, reduced viral load by 0.8–2.5 $\log_{10}$ at a dose of 30 mg/kg (11). Furthermore, passive transfer of 3BNC117 has been shown to delay viral rebound in HIV-infected individuals undergoing analytical treatment interruption (12). Two infusions of 30 mg/kg of antibody delayed viral rebound by 5–9 weeks whereas four infusions delayed for up to 19 weeks. This was significant compared to historical controls that typically rebounded less than 3 weeks after treatment interruption. Taken together, these studies highlight the safety and efficacy of passive transfer of HIV bNAbs for reduction of viremia and maintenance of viral suppression. Given this demonstrable potency against HIV in humans, bNAbs may also serve as effective tools in the context of HIV prophylaxis. In fact, a phase II clinical trial sponsored by the NIAID Vaccine Research Center (VRC) is currently recruiting participants to evaluate the safety and efficacy of VRC01 passive transfer in preventing HIV infection in high-risk, uninfected women in Sub-Saharan Africa (13). In addition, the VRC is recruiting participants for a phase I clinical trial to assess the safety of VRC01 passive transfer in infants less than 72 hours after birth at high risk for mother-to-child HIV transmission (14).

While these studies underscore the potential use for bNAbs in HIV therapy and prevention, passive transfer is likely an infeasible HIV vaccine approach. Given the short half-life (approximately 3 weeks) of antibodies *in vivo*, this approach would require continual readministration to maintain protective antibody concentrations. Previous clinical experience with preexposure prophylaxis indicates that consistent readministration generally reduces patient compliance (15). To lengthen the interval between doses, the fragment crystallizable (Fc) region of delivered antibodies can be engineered to exhibit enhanced affinity for neonatal Fc receptors (FcRn). This interaction facilitates antibody recycling through the endocytic salvage pathway in which endocytosed IgG binds FcRn within the early endosome and recycles back to the cell surface, thus preventing lysosomal degradation (16). Fc mutations that enhance interaction with FcRn, such as M428L/N434S (LS), T250Q/M428L (QL), and M252Y/S254T/T256E (YTE) have been shown to significantly improve antibody half-lives *in vivo* (17–19). In the NHP model, passive transfer of VRC01 harboring these mutations (VRC01-LS) resulted in a longer serum half-life, increased antibody concentration at mucosal surfaces, and enhanced protection against SHIV challenge compared to the wild-type antibody (20). A phase I clinical trial sponsored by the VRC is currently recruiting participants to assess the safety of passive transfer of VRC01-LS in healthy adults as the long-term immunogenicity of these engineered antibodies remains

unknown (21). Even considering the potential for longer intervals between doses, additional logistical requirements such as continual antibody production, temperature-controlled storage, and complex distribution networks, will further hinder the implementation of a passive transfer vaccine approach, particularly in developing countries where intervention is most needed. Therefore, other strategies must be explored for the generation of bNAb responses against HIV.

Historically, single- or limited-dose vaccines, such as for smallpox or polio, have represented the most effective eradication approaches against infectious diseases. Encouragingly, the bNAb-mediated protection observed in the aforementioned NHP studies and clinical trials occurred at modest antibody concentrations representing only about 1% of total circulating IgG1 (22). Therefore, substantial effort has been focused on the design of immunogens capable of eliciting HIV bNAb responses (23–25). Structural studies of bNAbs bound to their viral antigens have helped to elucidate major sites of Env vulnerability including the CD4bs, glycan-dependent epitopes in variable loops 1 and 2 (V1/V2) or variable loop 3 (V3), the gp120/gp41 interface, and the membrane proximal external region (MPER) (26–29). However, unique characteristics of bNAbs, such as high somatic mutation and a long heavy chain complementarity determining region 3 (CDRH3), make the use of existing vaccination strategies very challenging. For example, several studies have shown that vaccination against influenza elicits antibodies with mutation rates in the V-gene of approximately 5% (30,31); however, VRC01 demonstrates a mutation rate closer to 30% (32). This high level of affinity maturation is likely the result of continuous exposure to an evolving antigen which would be difficult to mimic with current immunogens (Fig. 1a). Therefore, many studies have examined the co-evolution of HIV with bNAbs in order to identify unmutated common and early ancestor antibodies and understand the antigens that direct bNAb development (33–36). This could inform potential prime-boost vaccination strategies involving sequential immunogens to direct bNAb elicitation (Fig. 1b). While such studies represent important conceptual advances for the field of immunogen design, these approaches would necessitate repeated administration, presenting challenges for patient compliance. In addition, the stochastic nature of antibody evolution and selection in each vaccine recipient makes it difficult to design regimens that will consistently and efficiently elicit bNAb responses in the vast majority of genetically diverse subjects.

Antibody Gene Transfer with Adeno-Associated Viral Vectors

As an alternative to traditional vaccination approaches, gene transfer has been used to produce HIV bNAbs *in vivo*. In this method, transgenes encoding the desired antibody are delivered to tissues, resulting in secretion and systemic expression of biologically active antibodies without the need for immunization (Fig. 1c). A variety of vector systems have been developed to deliver antibody transgenes including naked DNA and lentiviral vectors (37–39). Herein, we will focus on the use of adeno-associated virus (AAV) vectors for the delivery of bNAbs as they constitute the best-studied gene therapy vector for use in humans. Recently, Glybera, an AAV vector expressing lipoprotein lipase enzyme, was the first gene therapy to be approved for clinical use in Europe as treatment for familial lipoprotein lipase deficiency. In addition, AAV vectors have been employed to conduct over 100 gene therapy clinical trials for diseases ranging from cystic fibrosis and blindness to hemophila B. These

trials have proceeded in the absence of significant adverse events and some have yielded dramatic therapeutic efficacy (40). Beyond safety, AAV-based antibody gene transfer boasts many potential benefits as it bypasses dependence on humoral immunity and requires only a single dose for sustained expression of mature bNAbs. Moreover, AAV particles are resistant to high temperatures as well as wide-ranging pHs and can be stored in stable form for long periods of time without much loss of infectivity, enabling simpler distribution and implementation in resource-limited settings (41–43).

AAV is a small, icosahedral, non-enveloped virus belonging to the family *Parvoviridae* in the genus *Dependovirus* which depends on the presence of co-infecting helper viruses, such as adenovirus or herpesvirus, for productive infection. AAV packages a single-stranded DNA genome of approximately 4.7 kilobases (44) and is capable of infecting a variety of both non-dividing and dividing cell types including muscle, liver, and brain. Cellular tropism is defined by the identity of the viral capsid, of which hundreds have been described (45) and that fall into at least 9 serotypes. Upon infection, AAV escapes the endosomal network and traffics to the nucleus by a yet unknown mechanism where the 145 base pair AAV inverted terminal repeats (ITRs) located on either end of the genome self-prime second-strand synthesis by cellular polymerases. In the absence of helper virus, natural AAV infection utilizes the virally-encoded Rep proteins to facilitate site-specific integration at a locus on human chromosome 19 and establish latent infection (46–48).

Although over 70% of the human population are seropositive for AAV serotype 2 (AAV2) (49), natural AAV infection has not been definitively linked with any symptoms or disease. In 2015, Nault *et al* (50) identified AAV2 integrations in known cancer driver genes in a small subset (roughly 5%) of hepatocellular carcinomas, suggesting a potential pathogenic role for AAV2 in cancer. However, these results remain highly controversial as a second group re-analyzed the presented data and reached contradictory conclusions (51). While it is important to understand the risks associated with AAV, the insertional mutagenesis in question is likely dependent on viral Rep proteins which are excluded in AAV-based gene therapy vectors. The ITR sequences are the only cis-acting viral DNA elements necessary for replication and packaging; therefore, rAAV vectors consist only of the transgene of interest flanked by AAV2 ITR sequences. Rep and Cap proteins as well as necessary helper proteins are then provided in trans to produce encapsidated replication-incompetent rAAV in cell lines. Upon administration, the rAAV vector lacking Rep gene function can transduce cells where it persists as an episome composed of a series of head-to-tail concatemers of the recombinant genome in the nucleus of infected cells (52). Previous studies have demonstrated sustained expression following intramuscular (IM) injection of rAAV, with one study observing transgene expression in human skeletal muscle 10 years post administration (53–56). As a result of the highly vascularized nature of muscle tissue, transgenes introduced by IM injection can also be released into systemic circulation (57–59).

In 2002, Lewis *et al* were the first to demonstrate the feasibility of rAAV-vectored gene transfer to generate systemic expression of biologically active, full-length HIV bNAbs (60). Their vector consisted of two distinct promoters, each driving the expression of either the heavy or light chain gene of b12, an early-generation CD4bs-directed bNAb. Immunodeficient Rag1 mice given a single IM injection of rAAV1/b12 achieved serum

antibody concentrations as high as 8 μg/mL which persisted for the 6 months of the study. The b12 produced in these animals retained its biological activity against HIV-1 as shown in *in vitro* neutralization assays against primary and T-cell line adapted HIV-1 isolates.

Fang *et al* (61) were able to achieve superior full-length antibody concentrations following intra-hepatic AAV8 administration by engineering a bicistronic vector in which both the heavy and light chain genes were expressed from a single open reading frame. This vector utilized the 2A self-processing peptide derived from the foot-and-mouth disease virus which promotes ribosomal skipping of a glycyl-prolyl peptide bond at the C-terminus of the 2A peptide (62). This results in release of the polypeptide from the ribosome while allowing synthesis of the downstream peptide to continue. Incorporation of an optimized furin cleavage site upstream of the 2A sequence ensured that no residual amino acids from the 2A peptide remained on the expressed antibody (63). When the anti-VEGFR2 antibody DC101 was expressed from this vector, functional antibody was produced which maintained antigen binding *in vitro* and achieved highly efficient expression *in vivo*. A single dose of rAAV8/DC101 administered through the portal vein to nude mice led to serum antibody concentrations greater than 1000 μg/mL for 4 months (61,63). Given the limited carrying capacity of AAV vectors, the use of 2A peptide is substantially more space-efficient than dual-promoter configurations and was fundamental to the development of vectors capable of efficient full-length HIV bNAb expression.

AAV-Delivered Antibodies Confer Protection from HIV Infection

In 2009, Johnson *et al* (64) were the first to demonstrate that AAV-delivered antibody-like proteins conferred protection against a challenge infection. They engineered immunoadhesin molecules consisting of SIV gp120-specific variable heavy and variable light chains connected by a linker and joined to the constant heavy region of the rhesus macaque IgG2 molecule. These antibody-like proteins exhibited potent neutralization against SIV strains *in vitro* and were used to test protection following gene transfer with a self-complementary AAV (scAAV) vector. The genome of scAAV vectors is engineered to form an intramolecular double-stranded DNA template, allowing for more rapid expression with the drawback of reduced carrying capacity. Rhesus macaques given an intramuscular injection of scAAV1 expressing SIV-specific immunoadhesins achieved serum concentrations between 40 and 190 μg/mL 4 weeks after AAV administration. At this time, animals were challenged intravenously with a single dose of the SIV molecular clone SIVmac316 that was sufficient to infect all control animals. Six of the 9 macaques previously given scAAV encoding immunoadhesins were completely protected from infection as assessed by the lack of both Gag-specific antibodies and SIV RNA in the plasma. Those macaques expressing immunoadhesins that went on to become infected were found to have significant anti-transgene responses in the form of immunoadhesin-specific antibodies, which correlated with a sharp decline in immunoadhesin serum concentrations and loss of protection. This was the first study to demonstrate the efficacy of an AAV-based vaccine approach in a model of HIV transmission. However, their AAV vector configuration exhibited limited carrying capacity that was unable to accommodate full-length antibody sequences necessitating the use of non-natural immunoadhesin molecules.

Our lab has previously described the development of an optimized expression vector capable of inducing long-term, systemic expression of full-length, naturally-occurring human bNAbs (65,66). This vector utilizes the aforementioned 2A peptide to enable bicistronic expression of bNAb heavy and light chains. IM injection of rAAV8 expressing b12 into both immunocompetent and immunodeficient mouse strains led to antibody serum concentrations greater than 100 μg/mL for at least 52 weeks. In humanized mice, rAAV delivery of HIV bNAbs protected animals from a single intravenous challenge dose of the HIV molecular clone NL4-3. Control mice exhibited significant CD4 cell depletion whereas mice expressing b12 did not. Other HIV bNAbs including 2G12, 4E10 and 2F5 exhibited partial protection in which some mice maintained high CD4 cell counts and others experienced delayed depletion. To assess the concentration necessary to afford protection from intravenous challenge, we performed a titration experiment in which we administered decreasing doses of rAAV expressing b12 or VRC01 which ultimately required serum antibody concentrations of 34 μg/mL and 8.3 μg/mL respectively to afford protection (65). This was the first study to demonstrate highly efficient, sustained expression of full-length HIV bNAbs from a single intramuscular injection that resulted in protection from intravenous HIV challenge.

The majority of human HIV transmission occurs through sexual contact. Therefore, we utilized the same approach, termed vectored immunoprophylaxis (VIP), in the bone marrow-liver-thymus (BLT) humanized mouse model to assess the ability of rAAV-delivered HIV bNAbs to protect against mucosal transmission. When rAAV8 expressing VRC01 was administered intramuscularly to BLT mice, antibody concentrations reached 100 μg/mL in the serum and approximately 100 ng/mL in the vaginal mucosa. Four weeks after AAV administration, mice were subjected to weekly, nonabrasive, low-dose, intravaginal challenge of JR-CSF, a CCR5-tropic HIV molecular clone. Control mice became infected after an average of 4 to 5 challenges as measured by viral RNA in the plasma. Conversely, 5 of 8 BLT mice expressing VRC01 were completely protected following 15 consecutive challenges. The 3 mice that became infected required 13–15 challenges before plasma viremia could be detected. Interestingly, two of these infected mice exhibited escape from VRC01 using the identical amino acid substitutions within envelope. This result argues against absolute sterilizing immunity as the mechanism of bNAb mediated protection in this model given the requirement for viral replication to acquire escape mutations. We favor a model in which bNAbs protect by blunting viral replication sufficiently to extinguish most but not all infection events. Heterosexual transmission of HIV between humans is a fairly inefficient process occurring between 1 in 100 and 1 in 1000 exposures (67,68). Most often, successful HIV infection is the result of propagation of one viral strain, termed the transmitted/founder (T/F) virus. In order to determine if T/F strains were more resistant to antibody neutralization compared to lab-adapted strains, we performed a second repetitive mucosal challenge study using REJO.c, a CCR5-tropic T/F molecular clone. IM injection of rAAV expressing VRC07G54W, a more potent CD4bs-directed bNAb, led to antibody concentrations in the serum and vaginal mucosa comparable to that observed for VRC01, but conferred complete protection against 21 consecutive intravaginal challenges of REJO.c (66).

In 2015, Saunders *et al* (69) demonstrated that rAAV8 delivery of simianized broadly neutralizing antibodies protected non-human primates from mucosal SHIV infection. The study used the same rAAV expression vector we had previously developed. IM injection of AAV expressing simianized VRC07 resulted in serum antibody concentrations of 2.5 to 7.7 μg/mL 2 to 4 weeks following administration. However, antibody concentrations became undetectable by 9 weeks coinciding with an increase of anti-VRC07 responses. To circumvent this issue, the rhesus immune response was suppressed prior to rAAV administration using cyclosporine (CsA). This resulted in an average peak serum antibody concentration of 38.12 μg/mL across 6 macaques. Following cessation of CsA treatment, antibody concentrations decreased due to the appearance of anti-antibody responses. However, 3 of the 6 animals maintained serum antibody concentrations between 1 and 10 μg/mL for 16 weeks. Macaques given rAAV8/VRC07 and CsA treatment were challenged intrarectally with the CCR5-tropic SHIV-BaLP4 5.5 weeks after AAV administration. All control animals became infected, but 4 of the 6 macaques expressing VRC07 were protected. The 2 animals that became infected exhibited little to no VRC07 expression at the time of challenge.

A study by Fuchs *et al* (70) showed that long-term expression of anti-SIV antibodies could protect from successive intravenous challenges of SIVmac239. They used two AAV vector approaches to deliver full-length IgG1 antibodies derived from previously described SIV-specific immunoadhesin sequences. In the first approach, they used a traditional single-stranded rAAV vector to express the full-length antibody. In the second approach, they used scAAV in which the heavy and light chain genes were delivered on separate vectors. IM injection of AAV led to variable serum concentrations with no discernable difference between the two vector approaches. Most macaques exhibited a precipitous drop in antibody concentration that coincided with the development of anti-antibody responses. However, one animal achieved a serum concentration of 270 μg/mL which persisted for more than 2 years in the absence of anti-anti responses. Interestingly, this animal was the only one to resist 6 consecutive intravenous challenges with SIVmac239, the last of which was 10 intravenous infectious doses. While macaques with low-level antibody expression became infected, they did exhibit significantly lower SIV viral RNA during peak viremia and chronic phase infection. In addition, time from exposure to peak viremia was also significantly longer in these animals.

Lastly, Gardner *et al* (71) used a rAAV-based approach to deliver eCD4-Ig to NHPs in place of HIV bNAbs. This antibody-like protein consists of the immunoadhesin form of CD4 fused to a sulfopeptide mimic of CCR5 and demonstrates broad and potent neutralization of HIV-1 isolates *in vitro*. IM injection of rAAV expressing the rhesus macaque form of eCD4-Ig in conjunction with a second vector expressing a required sulfation enzyme resulted in serum concentrations that stabilized between 17–77 μg/mL for the last 10 weeks of the 40-week study. Beginning 8 weeks after rAAV administration, macaques were challenged intravenously with increasing doses of SHIV-AD8, none of which successfully infected macaques expressing eCD4-Ig. Interestingly, animals exhibited minimal anti-transgene responses indicating that eCD4-Ig may be less immunogenic than the simianized antibodies previously described. However, it remains to be seen whether the non-natural structure of this antibody-like protein may yet elicit anti-transgene responses over a longer period of

observation. In addition, the need for co-administration of a second AAV encoding an enzyme for sulfation of the CCR5 mimetic peptide may hinder the clinical development of this approach as it relies on efficient transduction of both vectors for effective neutralization to occur and might necessitate an evaluation of the clinical safety of each separate component prior to testing their combination in human subjects.

Potential Pitfalls of AAV-Vectored Antibody Gene Transfer

While sustained expression of HIV broadly neutralizing antibodies and antibody-like proteins is a promising strategy for HIV prevention, there are still many obstacles to widespread use in humans. For one, AAV has a limited carrying capacity of approximately 5 kilobases which restricts the potential transgenes that can be expressed with these vectors. The introduction of the short 2A peptide sequence was an important advance as it maximized available vector space by abrogating the need for a second promoter. Despite this advance, efforts to further enhance transgene expression or engineer regulatable systems are still hindered by carrying capacity. Self-complementary AAV (scAAV) vectors may represent a method for more rapid and efficient transgene expression compared to traditional single-stranded rAAV vectors, though they can only accommodate half the natural carrying capacity, making them impractical for delivery of full-length antibodies in a single scAAV (32). The improved expression kinetics of scAAV vectors *in vivo* are thought to result from the ability of these vectors to form an intramolecular double-stranded DNA structure which circumvents the rate-limiting step of second-strand synthesis following transduction. However, it is not yet clear whether the improved expression characteristics are in fact a function of their unique structure or a result caused by difficulty in accurately quantifying scAAV vector preparations (72). Regardless, ongoing efforts will be necessary to further optimize rAAV vector configurations to maximize *in vivo* transgene expression in patients.

Another potential area of concern is the prevalence of pre-existing immunity to AAV. Approximately 60–70% of the human population is seropositive for antibodies against capsid for AAV1 and 2 (49). The presence of these antibodies would be predicted to significantly inhibit AAV transduction and thus reduce the efficacy of any AAV-based treatment. However, several strategies exist to address this issue. First, a wide variety of AAV serotypes could be employed which have exhibited limited circulation in human populations. The AAV8 serotype was isolated from rhesus macaques and is advantageous for its propensity for muscle transduction (73), reduced immunogenicity (74) and lower prevalence of seropositive individuals (about 38%) in the population (49). However, sufficient cross-reactivity between AAV capsids exist such that some individuals will still exhibit neutralizing activity against AAV8. While patient sera can be pre-screened for neutralizing factors prior to rAAV administration, new strategies will need to be developed to combat pre-existing immunity. Significant effort is being focused on the discovery of novel capsids that can improve tropism for tissues of interest (75) and reduce reactivity with existing human antibodies. For example, Zinn *et al* (76) utilized in silico reconstruction to infer the evolutionary intermediates of existing AAV serotypes. This lead to the identification of Anc80, the predicted ancestral capsid sequence, which maintained structural and functional integrity. Importantly, antibodies raised against current AAV serotypes demonstrated little to no cross-reactivity with Anc80. Regardless of the capsid used,

antibodies will likely arise following administration thereby hindering repeated use in a single individual and highlighting the need to develop a repertoire of capsids for which pre-existing immunity is limited.

One of the major issues that has arisen from AAV studies in NHP models has been the elicitation of anti-transgene immune responses that in some cases abolish efficacy against viral challenge (64,69,70,77). It is important to note, however, that none of the transgenes delivered in these studies were native macaque proteins, despite substantial effort to optimize the molecules for use in NHP. For example, simianization of VRC07 was performed by transferring the complementarity-determining regions of human VRC07 onto homologous macaque germ line genes and engineering key somatic mutations within the framework regions. The heavy and light chain variable regions were then appended to macaque constant regions (69). While this simianized antibody maintained specificity and potency, it is uncertain how well it mimics native rhesus antibodies as it did not undergo immunological selection in macaques. Similarly, antibodies used by Fuchs *et al* (70) were originally isolated by phage display and then converted into full-length simian antibodies, again skipping the key negative selection process. There is recent evidence that naturally elicited SIV-specific macaque antibodies isolated from NHP by means similar to human bNAbs exhibit substantially better expression characteristics in the absence of detectable anti-antibody responses following rAAV administration (HIV Keystone meeting 2016, Mario Roederer). As AAV-vectored antibody gene transfer will be used to deliver only fully human antibodies that underwent selection by the human immune system, we hypothesize that they will exhibit reduced immunogeniticy than might have been predicted by published NHP studies. Importantly, individuals receiving infusions of VRC01 protein did not generate any detectable anti-antibody responses as long as 112 days post infusion (10). Therefore, it is unclear whether anti-antibody responses will be an area of concern in human patients.

While we do not believe that immunogenicity against the transgene will be widespread if rAAV/bNAb vectors are used at large, it is entirely possible that rare individuals may experience interactions that could not be predicted *a priori*. In these instances, it would be highly desirable to have a means of reversing antibody gene expression. Inducible rAAV vector systems may provide one such method for regulatable expression as transgene transcription is dependent upon the presence of a small molecule such as tetracycline, mifepristone, or rapamycin (78–82). In these systems, the small molecule allows for transcriptional activation domains to be recruited to a basal promoter driving transgene expression. In 2007, Fang *et al* (63) demonstrated the ability of a rapamycin-inducible rAAV vector to drive high-level expression of full-length antibodies *in vivo*. Using a dimerization-regulated system, they attained serum antibody concentrations greater than 1 mg/mL which rapidly returned to baseline after discontinuation of rapamycin treatment. However, these inducible systems present several challenges for clinical application in the context of VIP. First, they require expression of transcriptional activators that would be difficult to accommodate into a single rAAV vector along with a full-length antibody gene. Second, long-term expression of these heterologous proteins may be immunogenic in patients. In 2006, Murphy *et al* (83) described a drug-dependent inducible system that circumvented these issues as it did not depend on expression of heterologous transcriptional activation domains. Instead, they engineered the transgene of interest with a stop codon just

downstream of the site of translation initiation, thus preventing transgene expression. Subsequent treatment with aminoglycoside antibiotics and other nonantibiotic compounds resulted in stop codon read-through and a 60-fold induction of transgene expression. In a similar approach, Yen *et al* (84) engineered transgenes to contain the hammerhead Sm1 ribozyme sequence, a cis-acting RNA motif derived from *Schistosoma mansoni* that mediates self-cleavage of the mRNA transcript and prevents transgene expression. Upon administration of antisense oligonucleotides, they observed anywhere from 20–2000-fold induction of transgene expression depending on the inducer molecule used. As with the stop codon read-through approach, this system is more amenable to rAAV vectors as only short nucleic acid sequences are required. However, all of the inducible systems described necessitate continual adherence to a daily pill regimen and therefore pose no advantage to current anti-retroviral treatments. We favor a system in which transgene expression is on by default unless intervention is required. Analogous to inducible systems, drug-dependent repressible rAAV vectors have been engineered in which small molecules block transgene expression (85) and are only necessary in the rare subset of patients in which a negative reaction occurs. In a similar approach, lentiviral vectors encoding the Herpes Simplex thymidine kinase (HSVTK) gene have been used to allow for specific destruction of transduced cells upon ganciclovir administration (86). However, these drug-dependent repressible systems would share the drawbacks of limited carrying capacity and potential immunogenicity of necessary heterologous proteins. Additionally, destruction of skeletal muscle with HSVTK-encoding vectors would likely have devastating effects. A more advanced approach currently under development by our lab utilizes Cre/loxP recombination to specifically elicit transgene destruction (unpublished data). In this method, the initial rAAV vector used for antibody gene delivery contains several loxP sites flanking the antibody gene and other regulatory elements. Upon Cre administration to transduced cells, recombination of these sites will lead to degradation of the transgene and a drastic reduction in expression. These regulated approaches will be important to ensure the safety of antibody gene transfer in widespread use.

Improving Expression and Efficacy of AAV-Delivered Broadly Neutralizing Antibodies

We and others are continually working to improve the efficacy of AAV-mediated antibody gene transfer. One strategy is to improve the potency and breadth of the antibodies delivered. Since the characterization of the first major bNAbs (b12, 2G12, 2F5, 4E10, and Z13) in the early 90s, significant effort has been invested in the isolation and engineering of newer bNAbs with improved neutralization profiles. For example, PGDM1400, an HIV bNAb specific to the Env trimer apex, has exhibited a cross-clade neutralization coverage of 83% at a median half-maximal inhibitory concentration (IC_{50}) of 0.003 μg/mL according to *in vitro* neutralization panels (87). Similarly, Mark Connors' lab at NIAID has recently identified N6, a new CD4bs-directed bNAb more potent than VRC07 and broader in the range of envelope sequences it recognizes (unpublished data). These antibodies represent a significant improvement over early generation bNAbs such as the CD4bs-directed bNAb, b12 which demonstrated an approximate 34% cross-clade neutralization coverage at a median IC_{50} of 2.82 μg/mL (3). Previous studies indicate that bNAbs with better *in vitro*

neutralization characteristics can confer better protection *in vivo.* For example, the potent glycan-dependent bNAb, PGT121, was able to protect at an average serum antibody concentration approximately 100-fold lower than b12 in NHP models of mucosal SHIV transmission (88). However, *in vitro* neutralization potency is not always an accurate predictor of efficacy *in vivo* as evidenced by our experience with 2G12. Despite being well over the IC_{50} value of 2G12 for NL4-3, we observed only partial protection, perhaps suggesting a factor of "escapability" (i.e. the ease in which HIV can become resistant to a given antibody) as a new feature to consider when selecting bNAbs. As a consequence, newly discovered antibodies should be tested for their ability to confer protection *in vivo* in order to identify the best candidates for use in humans.

Delivery of AAV cocktails expressing combinations of HIV bNAbs may be advantageous to extend clade coverage and reduce potential for viral escape. However, we believe that prevention of HIV infection and reduction in transmission may be achievable through expression of a single bNAb provided it is sufficiently potent against most circulating strains of HIV. Similarly, single bNAb administration may be sufficient to maintain viral suppression as evidenced by the recent phase II clinical trial in which administration of 3BNC117 alone delayed viral rebound in suppressed patients following treatment interruption (12). Therefore, bNAb combinations may be particularly important in the context of HIV therapy as on-going viral replication produces quasi-species that may be differentially susceptible to any single bNAb. When combinations of bNAbs with varying epitope specificity were given by passive transfer to humanized mice, plasma viral load exhibited prolonged suppression compared to monotherapy (89). Interestingly, several mice receiving tri-mix therapy exhibited viral rebound due to the acquisition of viral escape variants whereas mice receiving penta-mix therapy suppressed viremia for the duration of the study. In 2016, Wagh *et al* assessed 15 different single bNAbs for neutralization potency against a panel of HIV clade C viruses in order to inform mathematical models of potential combinations (90). Certain 3 and 4 bNAb combinations were found to exhibit superior potency, breadth, complete neutralization, and active coverage compared to 2 bNAb combinations or any one bNAb alone. However, further *in vivo* studies will be necessary to determine the optimal combination of bNAbs for HIV therapy.

We are currently investigating additional methods to enhance the effectiveness of HIV bNAbs delivered by rAAV vectors. As previously mentioned with passive transfer studies, antibodies can be engineered to contain Fc mutations such as LS, QL, and YTE to selectively enhance the affinity of Fc for FcRn and significantly improve antibody half-life (17–19). It is unknown the extent to which FcRn-mediated antibody recycling contributes to the steady-state serum antibody concentration achieved following IM rAAV administration *in vivo*. This is important to determine as it will inform the design of optimal strategies to enhance bNAb expression. If recycling contributes substantially to steady-state concentrations, Fc mutations that enhance FcRn interactions may provide an important tool for enhancing both serum and mucosal antibody concentrations following rAAV administration. Translating such improvements to humans could enable the use of substantially reduced vector dosage to attain clinically relevant antibody concentrations, thereby improving both the cost-effectiveness and safety of VIP.

In addition to the FcRn, the Fc region mediates interactions with complement proteins and Fc receptors on innate immune cells to facilitate antibody effector functions including complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), and antibody-dependent cellular phagocytosis (ADCP). Growing evidence suggests a role for these antibody-mediated effector functions, particularly ADCC, in HIV protection and control. For example, the RV144 phase III clinical trial in Thailand demonstrated a 31.2% reduction in HIV infection among participants receiving the vaccine; this protection is thought to correlate with high levels of ADCC (91,92). Additionally, HIV controllers have been shown to exhibit higher levels of ADCC-mediating antibodies compared to viremic subjects (93). This interest in antibody-mediated effector functions has led to investigations of their contribution to the mechanism of bNAb-mediated protection. In the NHP model, mutations abolishing effector function activity were engineered into the Fc region of b12. The mutated bNAb exhibited a significantly diminished capacity to prevent SHIV infection compared to wild-type b12 indicating a role for effector functions in protection (94). Similar results were observed using mutant human-mouse chimeric antibodies in a mouse model of HIV entry (95). The relative importance of each effector function to bNAb efficacy and whether this contribution is similar across bNAbs of differing epitope specificity remains poorly understood. If Fc-mediated functions are shown to be important, then rAAV vectors can be used to delivery mutant bNAbs with enhanced effector function activity, potentially conferring protection superior to that of wild-type bNAbs and other available treatments.

Conclusion

AAV-vectored delivery of HIV broadly neutralizing antibodies and antibody-like proteins has led to long-term, systemic transgene expression that conferred protection from viral challenge in a variety of animal models. Given the apparent difficulty in eliciting bNAb responses through traditional vaccine approaches, vectored gene transfer may be a practical, effective strategy to prevent human HIV transmission. With the isolation of broader and more potent neutralizing antibodies as well as the potential for antibody engineering to enhance expression and effector function activity, it may be possible to further improve the protective efficacy of existing bNAbs. Anti-transgene responses following AAV administration are a potential area of concern as they have abrogated protection in NHP models; however, given the non-native nature of the molecules delivered in these studies it is uncertain whether analogous humoral responses will be elicited in human subjects receiving native human antibodies. Two phase I clinical trials have been planned to evaluate the safety of AAV-delivered HIV bNAbs. The first trial sponsored by the International Aids Vaccine Initiative recruited healthy adult men in the United Kingdom to receive a rAAV1 vector expressing PG9. This trial was scheduled to conclude in early 2016, however, the findings have not yet been published. The second trial sponsored by the VRC will test a rAAV8 vector expressing VRC07 in HIV infected patients. Results from these studies will undoubtedly shed light on the present controversy surrounding anti-antibody responses and is likely to reveal other potential challenges that will need to be overcome to enable widespread use of antibody gene transfer in humans.

Acknowledgments

A.B.B. is supported by the National Institutes for Drug Abuse (NIDA) Avenir New Innovator Award DP2DA040254, the MGH Transformative Scholars Program as well as funding from the Charles H. Hood Foundation. This independent research was supported by the Gilead Sciences Research Scholars Program in HIV. D.B. and A.B.B were supported by the National Institutes of Health (HHSN266200500035C) through a contract from the NIAID.

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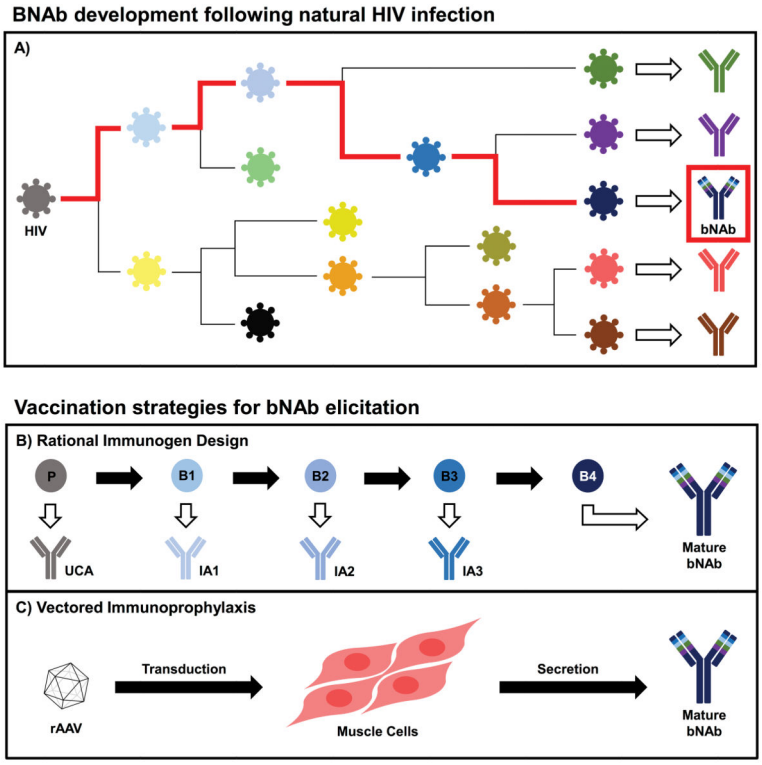

Figure 1.

Elicitation of bNAb responses for prevention of HIV infection. A) Following natural infection, HIV replication leads to viral diversification and development of mostly strain-specific humoral responses. In rare instances, however, bNAbs capable of cross-clade neutralization develop after several years of infection through continual affinity maturation with evolving Env epitopes (red path). B) By mimicking key intermediates of the natural bNAb developmental pathway, prime-boost vaccination strategies are hypothesized to elicit bNAb responses. In this approach, an initial prime (P) consisting of recombinant Env protein is first administered to interact with the unmutated common ancestor (UCA) of the desired bNAb. Subsequent booster doses (B1-4) would elicit intermediate ancestors (IA1-3) and direct antibody development toward the mature bNAb. However, this approach would require several doses and may not consistently produce the desired responses. C) Vectored immunoprophylaxis requires only a single intramuscular injection of recombinant adeno-associated virus (rAAV) vector encoding the antibody gene to bypass humoral immunity and elicit long-term, systemic expression of mature bNAbs. Following administration, the encapsidated rAAV vector transduces muscle cells and forms a stable double-stranded episome within the host cell nucleus. The transcriptionally-active episome drives expression of both the heavy and light chain antibody genes which self-assemble to form full-length bNAbs that are secreted into circulation.