



Engineering antibody-like inhibitors to prevent and treat HIV-1 infection

Matthew R. Gardner and Michael Farzan

Purpose of review

Here we discuss recently developed HIV-1 entry inhibitors that can target multiple epitopes on the HIV-1 envelope glycoprotein (Env), with an emphasis on eCD4-Ig. Some of these inhibitors are more potent and broader than any single antibody characterized to date. We also discuss the use of recombinant adeno-associated virus (rAAV) vectors as a platform for long-term expression of these inhibitors.

Recent findings

Much of the exterior of HIV-1 Env can be targeted by broadly neutralizing antibodies (bNAbs). Recent studies combine the variable regions or Fabs from different bNAbs, often with the receptor-mimetic components, to create broad, potent, and hard-to-escape inhibitors. rAAV vectors can express these inhibitors for years *in vivo*, highlighting their ability to prevent or treat HIV-1 infection.

Summary

By targeting multiple epitopes on Env, bispecific and antibody-like inhibitors can be broader and more potent than bNAbs. These inhibitors can provide long-term protection from, and perhaps suppression of, HIV-1 if they are administered by a delivery platform, like rAAV vectors, but only after rAAV limitations are addressed.

Keywords

adeno-associated virus gene therapy, alternative HIV vaccine, bispecific antibody, eCD4-Ig

INTRODUCTION

The HIV-1 envelope glycoprotein (Env) is a trimer of gp120/gp41 heterodimers that mediates viral entry. Env binds cellular CD4 and a tyrosine-sulfated coreceptor, primarily CCR5 or CXCR4, to translocate the viral capsid core across the host cell membrane (reviewed in [1]). As the sole viral protein expressed on the viral membrane, Env has been the main target of HIV-1 vaccine design. Although a conventional HIV-1 vaccine remains elusive, our understanding of broadly neutralizing antibodies (bNAbs) has significantly transformed immunogen design. Epitope mapping of bNAbs on Env has also shown that many distinct functional epitopes are exposed on Env (reviewed in [2]). The broadest and most potent bNAbs target the CD4-binding site, the base of the V3 loop (variable loop 3) around the N332 glycan, the apex of the V2 loop (variable loop 2), the membrane proximal external region (MPER), or the gp120/gp41 interface. Among these are the CD4-binding site antibodies VRC01 and 3BNC117 and the V3 glycan antibodies 10-1074 and PGT121. Individually, these bNAbs have clear therapeutic efficacy in rhesus macaques [3[•],4^{••},5–7]. Furthermore, VRC01, 3BNC117,

and 10-1074 have already shown promise in human clinical trials [8,9[•],10[•],11–13].

DESIGNING INHIBITORS THAT TARGET MULTIPLE ENV EPITOPES

Despite the numerous successes with bNAbs, there are obvious flaws with the use of single bNAb. No bNAb can neutralize all HIV-1 isolates, and most of the best characterized bNAbs fail to neutralize more than 30% of isolates tested with 80% inhibitory concentrations (IC_{80S}) > 20 μ g/ml. The two broadest

Department of Immunology and Microbiology, The Scripps Research Institute, Jupiter, Florida, USA

Correspondence to Matthew R. Gardner, Department of Immunology and Microbiology, The Scripps Research Institute, 130 Scripps Way, Jupiter, FL 33458, USA. Tel: +1 561 228 2109; e-mail: mgardner@scripps.edu

Curr Opin HIV AIDS 2017, 12:294–301

DOI:10.1097/COH.0000000000000367

This is an open access article distributed under the Creative Commons Attribution License 4.0 (CCBY), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

KEY POINTS

- The broadest and most potent HIV-1 inhibitors target multiple epitopes on Env or localize the inhibitor to the site of infection.
- Strategies that specifically target the CD4-binding and coreceptor-binding sites on Env can limit viral escape.
- eCD4-Ig is a very powerful inhibitor that has several attributes critical to preventing and treating HIV-1 infection.
- rAAV vectors can deliver protective concentrations of inhibitors like eCD4-Ig for years, potentially substituting for a conventional HIV-1 vaccine.

antibodies, N6 and 10E8, cover 98% of isolates assayed with 50% inhibitory concentrations (IC_{50} s) $< 50 \mu\text{g/ml}$ [14²²,15]. Even these exceptional cases underscore the fact that many isolates have already escaped these antibodies. Thus, treatment with a single antibody will rapidly select for mutations that render the antibody ineffective for several reasons. First is the high rate of mutation mediated by HIV-1 reverse transcriptase. Second, every bNAb has contact residues within their epitopes that are not necessary for Env function and not conserved. Third, because every Env has escaped similar antibodies in its recent past, there are easily accessible escape pathways requiring minimal mutations. Although less acute, escape remains a concern for prevention should resistant viruses become more frequent in an antibody-treated population. To overcome the weaknesses of individual bNAbs, studies have sought to optimize bNAb cocktails. Most cocktails combine antibodies that target distinct epitopes to increase breadth or that work synergistically to increase potency [16,17²³,18–20]. In a recent study, Wagh *et al.* [16] determined the best triple and quadruple antibody combinations against a panel of 200 clade C isolates. These types of studies suggest that at least three bNAbs will be necessary for preventing and treating HIV-1 infection.

Although targeting different epitopes on Env with multiple bNAbs is an effective strategy, efforts have been made to design single inhibitors that simultaneously target multiple sites on Env, including bispecific antibodies that combine two different bNAbs in a single construct (Fig. 1). For example, bispecific antibodies have been generated that combine VRC07 (CD4-binding site) with PGT121 (V3 glycan), 10E8 (MPER), or PG9-16 (V2 apex) and 10E8 with PG9-16 [21]. Recently, Bournazos *et al.* [22²⁴] demonstrated the utility of the IgG3 Fc for creating bispecific antibodies. To accommodate the distance

between two bNAb epitopes on Env, all the cysteines in the IgG3 hinge except two were mutated to serines. The authors concluded that the open IgG3 design could be used to increase the potency and breadth of other bNAbs. A separate study by Galimidi *et al.* [23] also showed that spacer length was crucial for a single inhibitor to target two epitopes. In this study, two Fabs were linked by dsDNA to form diFabs. Among the different diFabs generated, the hetero-diFab of PG16-3BNC60 with a 50-bp linker that targeted the CD4-binding site and V2 apex exhibited the greatest synergy. Together, these studies show that linkers of appropriate length are key to developing inhibitors that target two Env epitopes.

Although these traditional bispecific antibodies target two epitopes on Env, some of the most potent bispecific antibodies have taken a more unusual approach. The laboratory of David Ho has developed bispecific antibodies that target Env with one arm and an HIV-1 receptor or coreceptor with the other arm. Initially, single chain variable fragments (scFv) of PG9 or PG16 were combined with the heavy-chain N-terminus of ibalizumab (iMab), a humanized antibody that targets domain 2 of cellular CD4 [24]. Both bispecific antibodies, PG9-iMab and PG16-iMab, neutralized an entire panel of 118 HIV-1 isolates. Interestingly, these bispecific antibodies also neutralized HIV-1 isolates that were resistant to the component iMab, PG9, or PG16 antibodies. In a more recent study, Huang *et al.* [25²⁵] expanded their bispecific repertoire by generating numerous antibodies that combined one arm from a bNAb with either iMab or Pro140 (P140), an antibody that targets the coreceptor CCR5. Their most potent inhibitors combined the MPER antibody 10E8 with iMab (10E8/iMab) or P140 (10E8/P140) that had mean IC_{50} values of 2.0 and 1.0 ng/ml, respectively, when assayed against 118 HIV-1 isolates. 10E8/iMab exhibited 100% breadth with this panel, whereas 10E8/P140 had 99% coverage. These two studies demonstrate the effectiveness of localizing the Env-binding inhibitor directly to the site of infection.

INHIBITORS THAT TARGET THE CONSERVED CD4-BINDING AND CORECEPTOR-BINDING SITES

It remains unclear how effective bispecific antibodies will be if the virus can already escape from both arms of the antibody. Indeed, these antibodies are at best equivalent to bNAbs in patients harboring resistance to one arm. Consequently, other groups have sought to target two of the most conserved sites on Env, the CD4-binding, and coreceptor-binding

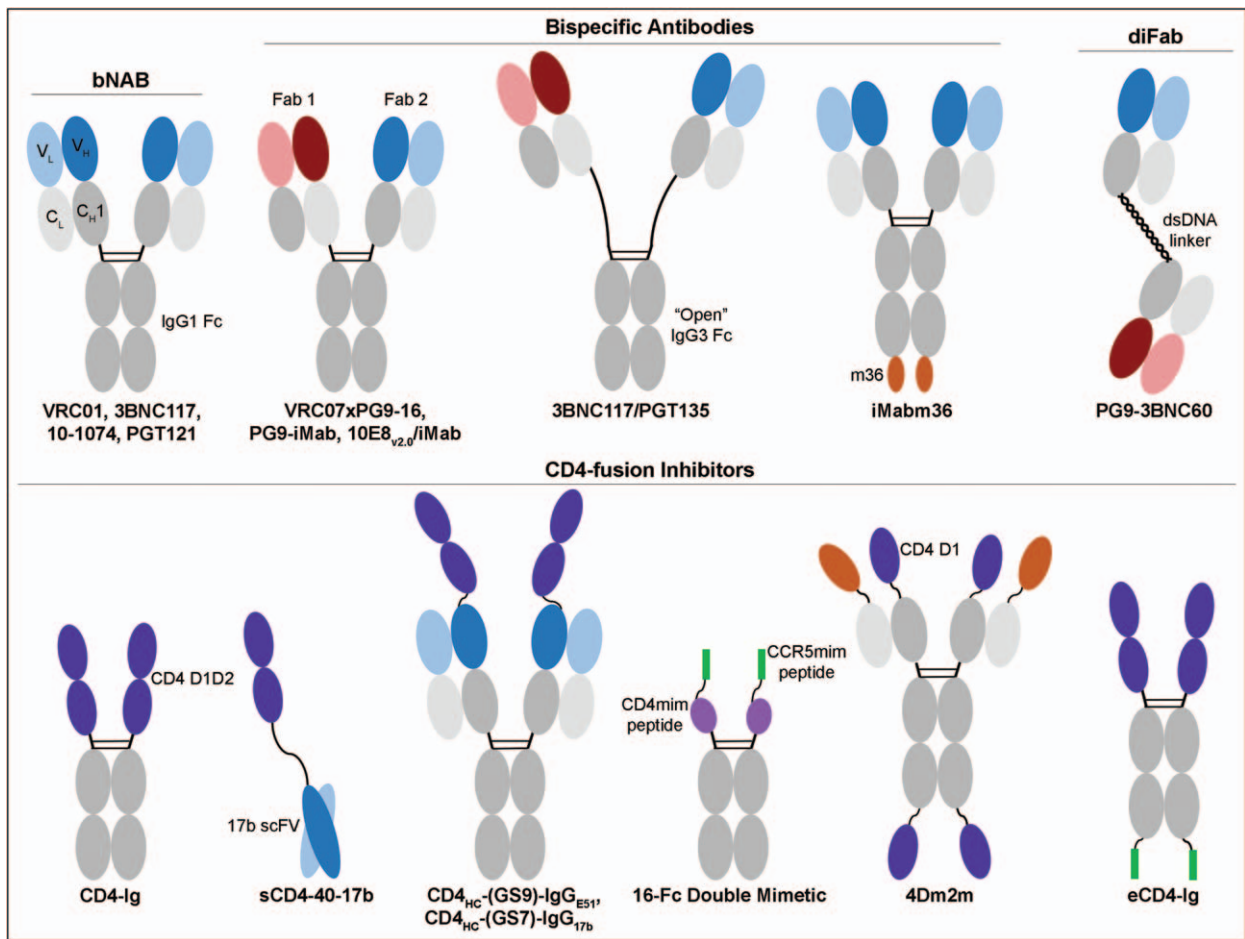


FIGURE 1. Designs of HIV-1 inhibitors that target multiple epitopes on HIV-1. Broadly neutralizing antibodies, bispecific antibodies, diFabs, and CD4-fusion inhibitors are represented, with examples named below each basic design. bNAb, broadly neutralizing antibody; CCR5mim, CCR5-mimetic sulfopeptide (green); CD4 D1D2, CD4 domains 1 and 2 (dark purple); CD4mim, CD4-mimetic (light purple); C_H1, constant heavy chain 1 (gray); C_L, constant light chain (light gray); dsDNA, double-stranded DNA; Fab, fragment antigen-binding; Fc, fragment crystallizable region (gray); IgG1, immunoglobulin 1; IgG3, immunoglobulin 3; scFv, single-chain variable fragment; V_H, variable heavy chain (blue or red); V_L, variable light chain (light blue or light red).

sites [26–31]. The conservation and functional roles of these sites make them ideal targets for inhibitor designs.

Soluble CD4 (sCD4) and its more bioavailable form, CD4-Ig, comprises the first two domains of CD4 (Fig. 1). In the case of CD4-Ig, these domains are fused to an IgG Fc. In addition, smaller CD4-like domains, based on CD4 domain 1 [32] or selected from a scorpion toxin scaffold [33], have been developed. To target the coreceptor-binding site, investigators have used constructs derived from CD4-induced (CD4i) antibodies, including 17b [34], E51 [35,36], and m36 [37], whose epitopes are more exposed in the presence of CD4 or sCD4. Some CD4i antibodies, such as E51, share with CCR5 a series of sulfated tyrosines critical to their ability to bind Env [36]. The first inhibitors to

target the receptor-binding sites fused sCD4 to the scFv of 17b (sCD4-40-17b) [38]. Similarly, West *et al.* [39] created sCD4 fusion constructs by fusing sCD4 to the N-terminus of E51 or 17b heavy chains of a full-length antibody. Two of these, CD4_{HC}-(GS9)-IgG_{E51} and CD4_{HC}-(GS7)-IgG_{17b}, were shown to be more potent than CD4-Ig. The importance of linker length was further highlighted by Quinlan *et al.* [40] who described a double-mimetic inhibitor that linked a sulfated CCR5-mimetic peptide and a CD4-mimetic peptide fused to an IgG1 Fc. The double mimetic peptide (16-Fc) derived its potency from simultaneously binding both the CD4-binding and coreceptor-binding sites on monomeric gp120. Recently, Chen *et al.* [41] characterized a potent bispecific fusion protein – 4Dm2m – that combined elements of a stable CD4 domain 1

(D1.22) with the antibody domain of m36. Sun *et al.* [42] took a different approach by fusing m36 to the C-terminus of iMab (iMabm36), thus creating an inhibitor that targeted the CD4i epitope and localized to CD4⁺ cells.

A key weakness of most of the inhibitors described is that one or both of their domains bind epitopes that contact variable Env residues. These unconserved contact residues serve as easy targets for viral escape. In contrast, our lab has developed eCD4-Ig [43^{***}], which is CD4-Ig with a short tyrosine-sulfated CCR5-mimetic peptide [44,45] appended to its C-terminus. Its Env binding sites are thus small, conserved receptor-binding regions. eCD4-Ig is broader than any HIV-1 antibody described, potently neutralizing all 73 HIV-1 isolates initially tested, regardless of clade or coreceptor preference, as well as all SIV and HIV-2 isolates assayed. These included many isolates resistant to CD4-Ig and to the CD4-binding site antibodies VRC01, 3BNC117, and NIH45–46. We have since shown that eCD4-Ig also neutralizes an entire 200 isolate clade C panel. Interestingly, eCD4-Ig utilizes both of its CCR5-mimetic sulfopeptides as well as one CD4 arm to bind Env and neutralize HIV-1 isolates. In addition to engaging Env, these sulfopeptides prevent CD4-Ig from enhancing infection at low concentrations or when cellular CD4 was limiting. Moreover, eCD4-Ig is harder to escape than antibodies. After more than 60 passages *in vitro* under conditions that readily elicited escape from NIH45–46 and CD4-Ig, we observed partial resistance but no escape from eCD4-Ig. Remarkably, the eCD4-Ig-selected swarm was fully resistant to CD4-Ig. The difficulty of escape and the inability to identify resistant isolates highlight the two key features of eCD4-Ig. First, HIV-1 has never encountered an inhibitor like eCD4-Ig and does not appear to have an accessible pathway for escape. Second, there appears to be a clear fitness cost for escape eCD4-Ig, likely because of the close similarity between eCD4-Ig and the native receptors of HIV-1.

Another advantage of eCD4-Ig is the size of its gene (~1.38 kb). This feature has allowed eCD4-Ig to be combined with viral vectors with tight limitations on transgene size, specifically recombinant adeno-associated virus (rAAV). We delivered a rhesus macaque version of eCD4-Ig (rh-eCD4-Ig) using rAAV vectors to four rhesus macaques [43^{***}]. The macaques expressed rh-eCD4-Ig for almost a year at 17–77 µg/ml. These levels of rh-eCD4-Ig were able to protect all four macaques from six escalating SHIV-AD8 challenges that infected all four control macaques, up to 16 times the 50% animal infectious dose of this virus. In a follow-up study, we demonstrated that low levels of rh-eCD4-Ig protected four

eCD4-Ig-inoculated animals from challenge doses of SIVmac239 that infected all eight control macaques. These studies show that a one-time inoculation with AAV-eCD4-Ig can protect from high doses of divergent, neutralization resistant viruses for at least 1 year after inoculation. Studies of rAAV with other proteins suggest that protective concentrations could last for 5 years or more. Thus, although work on conventional vaccines remains slow, effective, universal, and long-term protection from HIV-1 may be more quickly accessible with rAAV and eCD4-Ig.

OVERCOMING THE HURDLES OF RECOMBINANT ADENO-ASSOCIATED VIRUS VECTORS

Despite the potential of AAV-eCD4-Ig, there are concerns with rAAV vectors that must be addressed before human trials can be initiated. rAAV vectors have been examined for safety in numerous clinical trials and are currently being used to treat hemophilia [46,47]. These vectors do not replicate or integrate and are generally considered well tolerated. However, their small gene cassette size (about 5.0 kb) limits their applications. Full-length antibodies can fit into a single-stranded rAAV vector, either using two promoters or with an F2A peptide separating the heavy and light chains [48–50]. However, bispecific antibodies require two heavy-chain and light-chain arms to bind different epitopes. Thus, the use of bispecific antibodies with rAAV vectors would require at least two different vectors. eCD4-Ig itself easily fits into rAAV vectors. However, we have observed that TPST2, the enzyme necessary for sulfating the CCR5-mimetic peptides, is necessary for eCD4-Ig's full activity *in vivo*. Fortunately, TPST2 is only 1.14 kb, thus a single rAAV can accommodate both *eCD4-Ig* and *TPST2* genes. Although our first *in vivo* studies provided eCD4-Ig and TPST2 in different vectors, we are currently evaluating rAAV vectors that contain both genes.

There is also the concern that the host immune response will limit transduction efficiency from rAAV vectors (Fig. 2) [51–54]. For example, there is high preexisting immunity to typical rAAV vectors that use AAV1 (67%), AAV2 (72%), and AAV8 (38%) capsids [55]. Multiple groups are working on creating new AAV capsids for greater transduction either by rational design or directed evolution [56–59]. Similarly, rAAV vectors that enter transduced cells are exposed to capsid degradation [60] and TLR9 activation [61,62]. Some groups have observed that mutating Tyr, Ser, and Thr residues on the AAV capsids limit capsid phosphorylation that leads to degradation and thus increases transduction efficiency [63–66]. TLR9 signaling leads to the

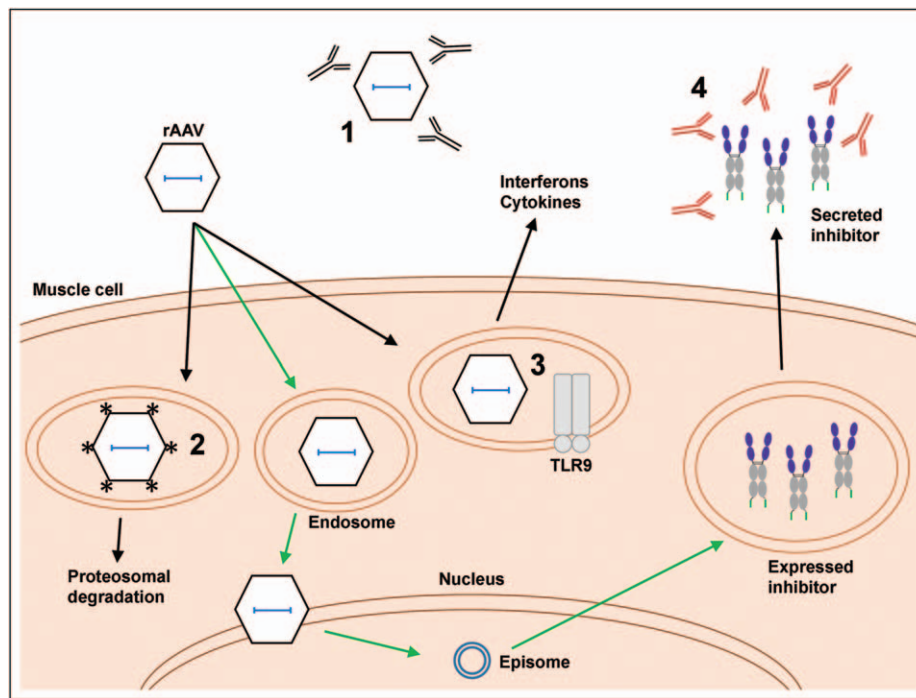


FIGURE 2. Summary of the current challenges for rAAV vector delivery. Preexisting immunity against AAV capsids will result in rAAV vectors that are neutralized before transducing the muscle cell. Upon entry into the muscle, rAAV vectors can get shuttled to the nucleus. However, rAAV capsids can be phosphorylated and targeted for premature degradation, or the TLR9 pathway can be activated leading to the production of interferons and proinflammatory cytokines. The rAAV vectors that reach the nucleus release their genomic material that forms episomes. The episome expresses the delivered inhibitor transgene that is secreted by muscle. The secreted inhibitors can also be targeted by host antibodies, limiting inhibitor levels in circulation, or by cytotoxic T cells, eliminating expressing cells entirely. Green arrows indicate pathway for production of inhibitor; black arrows indicate pathways that activate the immune response to limit transduction or inhibitor expression. rAAV, recombinant adeno-associated virus; TLR9, Toll-like receptor 9.

production of interferon and proinflammatory cytokines, which can promote killing of transduced cells by cytotoxic T cells. Others have shown effective rAAV inoculations using a TLR9 antagonist to prevent an innate immune response [62].

The expressed transgene is also an immune target. Indeed, we showed that two of four Rh macaques developed antibodies against rh-eCD4-Ig [43^{***}]. However, those two macaques were able to maintain rh-eCD4-Ig levels high enough to protect from SHIV challenges. In contrast, in our own studies, four bNAbs bearing rhesus macaque constant regions elicited very high levels of anti-bNAb antibodies that abrogated most expression and protection. Other groups have examined rAAV-delivered SIV and HIV-1 antibodies in macaques, again without as much success. Fuchs *et al.* [67] showed that expressed SIV antibodies 4L6 and 5L7 were targeted by antibodies in macaques that limited their efficacy against SIV challenges. In a recent study by Martinez-Navio *et al.* [68^{***}], eight of eight macaques that expressed rAAV-delivered rhesusized bNAbs 3BNC117, 10-1074, 10E8, 1NC9, and 8ANC195

developed anti-transgene antibodies that likely contributed to their low expression levels and lack of therapeutic efficacy. The severity of antitransgene response directly correlated to the amount of somatic hypermutation of each antibody. In a similar study, Saunders *et al.* [69] were able to overcome antibody responses to their rAAV-delivered simianized bNAbs by treating the Rh macaques with cyclosporin A. Mouse studies have examined micro-RNA binding sites in the gene cassette that limit antigen-presenting cell transduction [70], administration of CTLA4-Ig at the time of inoculation [71], or cotransduction of PD-L1 to limit host responses to immunogenic transgenes [71]. Another option would be to use rAAV8 vectors that target the liver as well as muscle tissue [72]. Liver transduction establishes tolerance to the expressed transgene and can be followed with a different AAV serotype for an additional boost. Studies of AAV-expressed antibodies demonstrate that expressed transgenes that are more unlike self are more likely to elicit an immune response [43^{***},67,68^{***},69,73]. Here again, eCD4-Ig has an advantage in that it is more self-like

than most bNAbs. Its only nonself regions are at the junctions of its three domains and the CCR5-mimetic peptide. The self-like quality of eCD4-Ig likely explains why antitransgene responses are modest and usually disappear over time.

A final hurdle to the use of rAAV as a vaccine alternative is the development of an effective kill switch. Although some high-risk or noncompliant individuals may benefit from AAV-eCD4-Ig, or AAV-expressed antibodies, the risk of a rare adverse event precludes wider use of this approach. Moreover, FDA (Food and Drug Administration) approval likely hinges on the existence of a kill switch that may even be critical to early human trials of an AAV-based vaccine. Intensive efforts to develop ways to permanently inactivate an AAV transgene are underway, and the problem appears considerably more tractable than the problem of developing a conventional vaccine. Moreover, these efforts can facilitate other uses of rAAV, for example, expressing antibodies that protect from diseases like malaria, or delivering biologics that are now being regularly injected.

CONCLUSION

The discovery and characterization of bNAbs over the last decade has generated numerous inhibitors that can potently neutralize many HIV-1 isolates. These inhibitors have significant advantages over single antibody therapy in that they can simultaneously target more than one epitope on Env, localize the inhibitor to the site of entry, or limit pathways of escape by binding conserved sites on Env. rAAV vectors appear to be a viable way to express some of these inhibitors, and these vectors can provide long-term protection from HIV-1. However, more research on rAAV gene delivery is needed to overcome its current limitations, and a kill switch would likely be required if this approach is to be used broadly. Improving these engineered inhibitors and overcoming the limitation of rAAV should be high-priority goals because this approach can demonstrably provide sustained, effective, and universal protection from HIV-1.

Acknowledgements

The authors would like to thank Meredith Gardner, Ina Fetzer, and Christoph Fellingner for their comments and edits of the article. The authors would also like to thank Meredith Gardner for her help with the figures.

Financial support and sponsorship

M.R.G. is supported by the NIAID Ruth L. Kirschstein NRSA F32 AI122980. M.F. is supported by the National Institutes of Health grants P01 AI100263, R37

AI091476, and by the Bill and Melinda Gates Foundation OPP1132169.

Conflicts of interest

M.R.G. and M.F. both are cofounders and part-owners of Emmune, Inc. and own patents based on eCD4-Ig-related technologies.

REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

1. Wilen CB, Tilton JC, Doms RW. HIV: cell binding and entry. *Cold Spring Harb Perspect Med* 2012; 2:a006866.
 2. Burton DR, Mascola JR. Antibody responses to envelope glycoproteins in HIV-1 infection. *Nat Immunol* 2015; 16:571–576.
 3. Liu J, Ghneim K, Sok D, *et al.* Antibody-mediated protection against SHIV ■ challenge includes systemic clearance of distal virus. *Science* 2016; 353:1045–1049.
- This study highlights mechanisms that bNAbs use for protecting rhesus macaques from SHIV infection.
4. Gautam R, Nishimura Y, Pegu A, *et al.* A single injection of anti-HIV-1 ■ antibodies protects against repeated SHIV challenges. *Nature* 2016; 533:105–109.
- In this study, Rh macaques were given a single infusion of a bNAb that provided protection from SHIV challenges for up to 23 weeks in some animals. These results show that a single bNAb infusion could be effective for months after passive infusion.
5. Ko S-Y, Pegu A, Rudicell RS, *et al.* Enhanced neonatal Fc receptor function improves protection against primate SHIV infection. *Nature* 2014; 514:642–645.
 6. Shingai M, Nishimura Y, Klein F, *et al.* Antibody-mediated immunotherapy of macaques chronically infected with SHIV suppresses viraemia. *Nature* 2013; 503:277–280.
 7. Barouch DH, Whitney JB, Moldt B, *et al.* Therapeutic efficacy of potent neutralizing HIV-1-specific monoclonal antibodies in SHIV-infected rhesus monkeys. *Nature* 2013; 503:224–228.
 8. Caskey M, Schoofs T, Gruell H, *et al.* Antibody 10–1074 suppresses viremia in HIV-1-infected individuals. *Nat Med* 2017. [Epub ahead of print]
 9. Scheid JF, Horwitz JA, Bar-On Y, *et al.* HIV-1 antibody 3BNC117 suppresses ■ viral rebound in humans during treatment interruption. *Nature* 2016; 535:556–560.
- This study highlights the ability for the CD4-binding site bNAb 3BNC117 to suppress HIV-1 rebound for 5–9 weeks after patients were released from antiretroviral drugs.
10. Bar KJ, Sneller MC, Harrison LJ, *et al.* Effect of HIV antibody VRC01 on viral ■ rebound after treatment interruption. *N Engl J Med* 2016; 375:2037–2050.
- This study highlights the ability for the CD4-binding site bNAb VRC01 to suppress HIV-1 rebound for 4–5 weeks after patients were released from antiretroviral drugs.
11. Lynch RM, Boritz E, Coates EE, *et al.* Virologic effects of broadly neutralizing antibody VRC01 administration during chronic HIV-1 infection. *Sci Transl Med* 2015; 7:319ra206–1319ra.
 12. Ledgerwood JE, Coates EE, Yamshchikov G, *et al.* Safety, pharmacokinetics and neutralization of the broadly neutralizing HIV-1 human monoclonal antibody VRC01 in healthy adults. *Clin Exp Immunol* 2015; 182: 289–301.
 13. Caskey M, Klein F, Lorenzi JCC, *et al.* Viraemia suppressed in HIV-1-infected humans by broadly neutralizing antibody 3BNC117. *Nature* 2015; 522: 487–491.
 14. Huang J, Kang Byong H, Ishida E, *et al.* Identification of a CD4-binding-site ■ antibody to HIV that evolved near-pan neutralization breadth. *Immunity* 2016; 45:1108–1121.
- This study identifies and characterizes the CD4-binding site bNAb N6, a very potent bNAb with tremendous breadth, covering 98% of the isolates tested. N6 may make a useful component of future bispecific antibodies and other HIV-1 inhibitors.
15. Huang J, Ofek G, Laub L, *et al.* Broad and potent neutralization of HIV-1 by a gp41-specific human antibody. *Nature* 2012; 491:406–412.
 16. Wagh K, Bhattacharya T, Williamson C, *et al.* Optimal combinations of broadly neutralizing antibodies for prevention and treatment of HIV-1 clade C infection. *PLoS Pathog* 2016; 12:e1005520.

17. Gardner MR, Fellingner CH, Prasad NR, *et al.* CD4-induced antibodies promote association of the HIV-1 envelope glycoprotein with CD4-binding site antibodies. *J Virol* 2016; 90:7822–7832.
- This study demonstrates how CD4-induced antibodies can promote binding to envelope (Env) of CD4-binding site bNAbs.
18. Klein F, Halper-Stromberg A, Horwitz JA, *et al.* HIV therapy by a combination of broadly neutralizing antibodies in humanized mice. *Nature* 2012; 492:118–122.
19. Doria-Rose NA, Louder MK, Yang Z, *et al.* HIV-1 neutralization coverage is improved by combining monoclonal antibodies that target independent epitopes. *J Virol* 2012; 86:3393–3397.
20. Zwick MB, Wang M, Poignard P, *et al.* Neutralization synergy of human immunodeficiency virus type 1 primary isolates by cocktails of broadly neutralizing antibodies. *J Virol* 2001; 75:12198–12208.
21. Asokan M, Rudicell RS, Louder M, *et al.* Bispecific antibodies targeting different epitopes on the HIV-1 envelope exhibit broad and potent neutralization. *J Virol* 2015; 89:12501–12512.
22. Bournazos S, Gazumyan A, Seaman Michael S, *et al.* Bispecific anti-HIV-1 antibodies with enhanced breadth and potency. *Cell* 2016; 165:1609–1620.
- This study highlights useful properties of an 'open' IgG3 fragment crystallizable region for designing bispecific antibodies.
23. Galimidi RP, Klein Joshua S, Politzer Maria S, *et al.* Intra-spike crosslinking overcomes antibody evasion by HIV-1. *Cell* 2015; 160:433–446.
24. Pace CS, Song R, Ochsenbauer C, *et al.* Bispecific antibodies directed to CD4 domain 2 and HIV envelope exhibit exceptional breadth and picomolar potency against HIV-1. *Proc Natl Acad Sci U S A* 2013; 110:13540–13545.
25. Huang Y, Yu J, Lanzi A, *et al.* Engineered bispecific antibodies with exquisite HIV-1-neutralizing activity. *Cell* 2016; 165:1621–1631.
- This study characterizes bispecific antibodies that use one arm to target Env and the second arm to target an HIV-1 receptor or coreceptor. These antibodies are some of the most potent antibodies described to date.
26. Wyatt R, Sodroski J. The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. *Science* 1998; 280:1884–1888.
27. Rizzuto CD, Wyatt R, Hernández-Ramos N, *et al.* A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding. *Science* 1998; 280:1949–1953.
28. Kwong PD, Wyatt R, Robinson J, *et al.* Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* 1998; 393:648–659.
29. Olshevsky U, Helseth E, Furman C, *et al.* Identification of individual human immunodeficiency virus type 1 gp120 amino acids important for CD4 receptor binding. *J Virol* 1990; 64:5701–5707.
30. Cordonnier A, Montagnier L, Emerman M. Single amino-acid changes in HIV envelope affect viral tropism and receptor binding. *Nature* 1989; 340:571–574.
31. Kowalski M, Potz J, Basirpour L, *et al.* Functional regions of the envelope glycoprotein of human immunodeficiency virus type 1. *Science* 1987; 237:1351–1355.
32. Chen W, Feng Y, Gong R, *et al.* Engineered single human CD4 domains as potent HIV-1 inhibitors and components of vaccine immunogens. *J Virol* 2011; 85:9395–9405.
33. Quinlan BD, Gardner MR, Joshi VR, *et al.* Direct expression and validation of phage-selected peptide variants in mammalian cells. *J Biol Chem* 2013; 288:18803–18810.
34. Thali M, Moore JP, Furman C, *et al.* Characterization of conserved human immunodeficiency virus type 1 gp120 neutralization epitopes exposed upon gp120-CD4 binding. *J Virol* 1993; 67:3978–3988.
35. Xiang S-H, Wang L, Abreu M, *et al.* Epitope mapping and characterization of a novel CD4-induced human monoclonal antibody capable of neutralizing primary HIV-1 strains. *Virology* 2003; 315:124–134.
36. Choe H, Li W, Wright PL, *et al.* Tyrosine sulfation of human antibodies contributes to recognition of the CCR5 binding region of HIV-1 gp120. *Cell* 2003; 114:161–170.
37. Chen W, Zhu Z, Feng Y, Dimitrov DS. Human domain antibodies to conserved sterically restricted regions on gp120 as exceptionally potent cross-reactive HIV-1 neutralizers. *Proc Natl Acad Sci U S A* 2008; 105:17121–17126.
38. Lagenaur LA, Villarroel VA, Bundoc V, *et al.* sCD4-17b bifunctional protein: Extremely broad and potent neutralization of HIV-1 Env pseudotyped viruses from genetically diverse primary isolates. *Retrovirology* 2010; 7:11.
39. West AP, Galimidi RP, Foglesong CP, *et al.* Evaluation of CD4–CD4i antibody architectures yields potent, broadly cross-reactive anti-human immunodeficiency virus reagents. *J Virol* 2010; 84:261–269.
40. Quinlan BD, Joshi VR, Gardner MR, *et al.* A double-mimetic peptide efficiently neutralizes HIV-1 by bridging the CD4- and coreceptor-binding sites of gp120. *J Virol* 2014; 88:3353–3358.
41. Chen W, Feng Y, Prabakaran P, *et al.* Exceptionally potent and broadly cross-reactive, bispecific multivalent HIV-1 inhibitors based on single human CD4 and antibody domains. *J Virol* 2014; 88:1125–1139.
42. Sun M, Pace CS, Yao X, *et al.* Rational design and characterization of the novel, broad and potent bispecific HIV-1 neutralizing antibody iMabm36. *J Acquir Immune Defic Syndr* 2014; 66:473–483.
43. Gardner MR, Kattenhorn LM, Kondur HR, *et al.* AAV-expressed eCD4-Ig provides durable protection from multiple SHIV challenges. *Nature* 2015; 519:87–91.
- This study characterizes eCD4-Ig, a broad and potent HIV-1 entry inhibitor that binds both the CD4-binding and coreceptor-binding sites. Recombinant adeno-associated virus delivered eCD4-Ig demonstrated complete efficacy *in vivo*, protecting all four inoculated Rh macaques from multiple escalating and high-dose SHIV challenges.
44. Chiang JJ, Gardner MR, Quinlan BD, *et al.* Enhanced recognition and neutralization of HIV-1 by antibody-derived CCR5-mimetic peptide variants. *J Virol* 2012; 86:12417–12421.
45. Dorfman T, Moore MJ, Guth AC, *et al.* A tyrosine-sulfated peptide derived from the heavy-chain CDR3 region of an HIV-1-neutralizing antibody binds gp120 and inhibits HIV-1 infection. *J Biol Chem* 2006; 281:28529–28535.
46. Nathwani AC, Reiss UM, Tuddenham EGD, *et al.* Long-term safety and efficacy of factor IX gene therapy in hemophilia B. *N Engl J Med* 2014; 371:1994–2004.
47. Jiang H, Pierce GF, Ozelo MC, *et al.* Evidence of multiyear factor IX expression by AAV-mediated gene transfer to skeletal muscle in an individual with severe hemophilia B. *Mol Ther* 2006; 14:452–455.
48. Fang J, Qian J-J, Yi S, *et al.* Stable antibody expression at therapeutic levels using the 2A peptide. *Nat Biotechnol* 2005; 23:584–590.
49. Lewis AD, Chen R, Montefiori DC, *et al.* Generation of neutralizing activity against human immunodeficiency virus type 1 in serum by antibody gene transfer. *J Virol* 2002; 76:8769–8775.
50. Fuchs SP, Martinez-Navio JM, Gao G, Desrosiers RC. Recombinant AAV vectors for enhanced expression of authentic IgG. *PLoS One* 2016; 11:e0158009.
51. Kotterman MA, Yin L, Strazzeri JM, *et al.* Antibody neutralization poses a barrier to intravitreal adeno-associated viral vector gene delivery to non-human primates. *Gene Ther* 2015; 22:116–126.
52. Hareendran S, Balakrishnan B, Sen D, *et al.* Adeno-associated virus (AAV) vectors in gene therapy: immune challenges and strategies to circumvent them. *Rev Med Virol* 2013; 23:399–413.
53. Rogers G, Martino A, Aslanidi G, *et al.* Innate immune responses to AAV vectors. *Front Microbiol* 2011; 2:194.
54. Calcedo R, Wilson J. Humoral immune response to AAV. *Front Immunol* 2013; 4:341.
55. Boutin S, Monteilhet V, Veron P, *et al.* Prevalence of serum IgG and neutralizing factors against adeno-associated virus (AAV) types 1, 2, 5, 6, 8, and 9 in the healthy population: implications for gene therapy using AAV vectors. *Hum Gene Ther* 2010; 21:704–712.
56. Zinn E, Pacouret S, Khaychuk V, *et al.* In silico reconstruction of the viral evolutionary lineage yields a potent gene therapy vector. *Cell Rep* 2015; 12:1056–1068.
57. Davis AS, Federici T, Ray WC, *et al.* Rational design and engineering of a modified adeno-associated virus (AAV1)-based vector system for enhanced retrograde gene delivery. *Neurosurgery* 2015; 76:216–225.
58. Lochrie MA, Tatsuno GP, Christie B, *et al.* Mutations on the external surfaces of adeno-associated virus type 2 capsids that affect transduction and neutralization. *J Virol* 2006; 80:821–834.
59. Mao Y, Wang X, Yan R, *et al.* Single point mutation in adeno-associated viral vectors-DJ capsid leads to improvement for gene delivery *in vivo*. *BMC Biotechnol* 2016; 16:1.
60. Zhong L, Li B, Jayandharan G, *et al.* Tyrosine-phosphorylation of AAV2 vectors and its consequences on viral intracellular trafficking and transgene expression. *Virology* 2008; 381:194–202.
61. Zhu J, Huang X, Yang Y. The TLR9-MyD88 pathway is critical for adaptive immune responses to adeno-associated virus gene therapy vectors in mice. *J Clin Invest* 2009; 119:2388–2398.
62. Martino AT, Suzuki M, Markusic DM, *et al.* The genome of self-complementary adeno-associated viral vectors increases Toll-like receptor 9-dependent innate immune responses in the liver. *Blood* 2011; 117:6459–6468.
63. Zhong L, Li B, Mah CS, *et al.* Next generation of adeno-associated virus 2 vectors: point mutations in tyrosines lead to high-efficiency transduction at lower doses. *Proc Natl Acad Sci U S A* 2008; 105:7827–7832.
64. Martino AT, Basner-Tschakarjan E, Markusic DM, *et al.* Engineered AAV vector minimizes *in vivo* targeting of transduced hepatocytes by capsid-specific CD8⁺ T cells. *Blood* 2013; 121:2224–2233.
65. Gabriel N, Hareendran S, Sen D, *et al.* Bioengineering of AAV2 capsid at specific serine, threonine, or lysine residues improves its transduction efficiency *in vitro* and *in vivo*. *Hum Gene Ther Methods* 2013; 24:80–93.
66. Aslanidi GV, Rivers AE, Ortiz L, *et al.* Optimization of the capsid of recombinant adeno-associated virus 2 (AAV2) vectors: the final threshold? *PLoS One* 2013; 8:e59142.
67. Fuchs SP, Martinez-Navio JM, Piatak M Jr, *et al.* AAV-delivered antibody mediates significant protective effects against SIVmac239 challenge in the absence of neutralizing activity. *PLoS Pathog* 2015; 11:e1005090.
68. Martinez-Navio JM, Fuchs SP, Pedreño-López S, *et al.* Host anti-antibody responses following adeno-associated virus-mediated delivery of antibodies against HIV and SIV in Rhesus Monkeys. *Mol Ther* 2016; 24:76–86.
- This study highlights how anti-transgene responses can limit expression of secreted antibodies in rhesus macaques. The authors also show that antibodies with larger amounts of somatic hypermutation can lead to greater anti-transgene responses.

69. Saunders KO, Pegu A, Georgiev IS, *et al.* Sustained delivery of a broadly neutralizing antibody in nonhuman primates confers long-term protection against simian/human immunodeficiency virus infection. *J Virol* 2015; 89:5895–5903.
70. Majowicz A, Maczuga P, Kwikkers KL, *et al.* Mir-142-3p target sequences reduce transgene-directed immunogenicity following intramuscular adeno-associated virus 1 vector-mediated gene delivery. *J Gene Med* 2013; 15:219–232.
71. Adriouch S, Franck E, Drouot L, *et al.* Improved immunological tolerance following combination therapy with CTLA-4/Ig and AAV-mediated PD-L1/2 muscle gene transfer. *Front Microbiol* 2011; 2:199.
72. Lu Y, Song S. Distinct immune responses to transgene products from rAAV1 and rAAV8 vectors. *Proc Natl Acad Sci U S A* 2009; 106:17158–17162.
73. Johnson PR, Schnepf BC, Zhang J, *et al.* Vector-mediated gene transfer engenders long-lived neutralizing activity and protection against SIV infection in monkeys. *Nat Med* 2009; 15:901–906.