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_{80}s) > 20 μg/ml. The two broadest
antibodies, N6 and 10E8, cover 98% of isolates assayed with 50% inhibitory concentrations (IC50s) < 50 μg/ml [14**,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 [16,17*,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, Bournaozos et al. [22*] 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. [25**] 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 IC50 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
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 bp, 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
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 bNAb 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 bNAb 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 bNAb by treating the Rh macaques with cyclosporin A. Mouse studies have examined microRNA 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 bNAb. 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 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 bNAb 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.

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**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.

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