Published in final edited form as:

Sci Transl Med. 2019 July 24; 11(502): . doi:10.1126/scitranslmed.aau5409.

AAV-delivered eCD4-Ig protects rhesus macaques from high-dose SIVmac239 challenges

Matthew R. Gardner^{1,*,#}, Christoph H. Fellinger^{1,*}, Lisa M. Kattenhorn^{2,†}, Meredith E. Davis-Gardner¹, Jesse A. Weber¹, Barnett Alfant¹, Amber S. Zhou¹, Neha R. Prasad¹, Hema R. Kondur¹, Wendy A. Newton³, Kim L. Weisgrau³, Eva G. Rakasz³, Jeffrey D. Lifson⁴, Guangping Gao^{5,6}, Nancy Schultz-Darken³, Michael Farzan^{1,#}

¹Department of Immunology and Microbiology, The Scripps Research Institute, Jupiter, FL 33458, USA

²Department of Microbiology and Immunobiology, Harvard Medical School, New England Primate Research Center, Southborough, MA 01772, USA

³Wisconsin National Primate Research Center, University of Wisconsin, Madison, WI 53715 USA

⁴AIDS and Cancer Virus Program, Frederick National Laboratory for Cancer Research, Frederick, MD 21702, USA

⁵The Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA 01605, USA

⁶Department of Microbiology and Physiological Systems, University of Massachusetts Medical School, Worcester, MA, 01655, USA

Abstract

[#]To whom correspondence should be addressed: Matthew R. Gardner (mgardner@scripps.edu).

[†]Current address: Novartis Institutes for Biomedical Research, Cambridge, MA 02139, USA

^{*}Contributed equally

Author contributions: M.R.G. and M.F. conceived the study. M.R.G., C.H.F., L.M.K., and M.F. designed the experiments. M.R.G., C.H.F., L.M.K., M.E.D.-G., J.A.W., B.A., A.S.Z., N.R.P., and H.R.K. performed the experiments. L.M.K. conducted the non-human primate studies at NEPRC. W.A.N. and N.S.-D. conducted the non-human primate studies at WNPRC. E.G.R. and K.L.W. prepared reagents and processed animal samples for the non-human primate study at WNPRC. G.G. oversaw production of AAV1 vectors and screening for AAV1 seropositivity. J.D.L. oversaw viral load quantification experiments. M.R.G., J.D.F., and M.F. analyzed the data. M.R.G. and M.F. wrote the manuscript with input and approval from the other co-authors.

Overline: HIV

Supplemental Materials

Figure S1. AAV1-delivered rh-eCD4-Ig^{I39N} affords complete protection from high-dose challenges with SHIV-AD8.

Figure S2. Functional characterization of rh-eCD4-Ig^{I39N} expressed from an AAV gene cassette.

Figure S3. *In vivo* rh-eCD4-Ig^{I39N} concentrations plotted against number of SIVmac239 challenges until infection.

Figure S4. Protection from SIVmac239 is not due to a xenogenic response to the AAV1 vector.

Figure S5. Alignment of full-length SIVmac239 gp160 sequences cloned from infected macaques.

Figure S6. Sensograms of SIVmac239 gp120 variants binding rh-CD4-Ig and rh-CD4-Ig^{I39N}.

Data Table 1.

Competing interests: M.R.G. and M.F. are co-founders of Emmune, Inc., a company that is developing technologies for eCD4-Ig and AAV-eCD4-Ig. M.R.G. and M.F. are inventors on patent application no. 15/140,919 submitted on 28 April 2016 by The Scripps Research Institute that covers the development of AAV-delivered eCD4-Ig. M.R.G. and M.F. are inventors on patent application no. 62/645,903 submitted on 21 March 2019 by the Scripps Research Institute that covers improvements to eCD4-Ig proteins.

Data and materials availability: All data associated with this study are present in the paper or Supplementary Materials.

A number of simian and simian human immunodeficiency viruses (SIV and SHIV) have been used to assess the efficacy of HIV-1 vaccine strategies. Among these, SIVmac239 is considered among the most stringent because, unlike SHIV models, its full genome has co-evolved in its macaque host and its tier 3 envelope glycoprotein (Env) is exceptionally hard to neutralize. Here we investigated the ability of eCD4-Ig, an antibody-like entry inhibitor that emulates the HIV-1 and SIV receptor and coreceptor, to prevent SIVmac239 infection. We show that rh-eCD4-Ig^{I39N} expressed by recombinant adeno-associated virus (AAV) vectors afforded four rhesus macaques complete protection from high-dose SIVmac239 challenges that infected all eight control macaques. However, rh-eCD4-Ig^{I39N}-expressing macaques eventually succumbed to serial escalating challenge doses that were 2-, 8-, 16- and 32-times the challenge doses that infected the control animals. Despite receiving greater challenge doses, these macaques had significantly lower peak and post-peak viral loads than the control group. Virus isolated from three of four macaques showed evidence of strong immune pressure from rh-eCD4-Ig^{I39N}, with mutations located in the CD4-binding site, which in one case exploited a point-mutation difference between rh-eCD4-Ig^{I39N} and rhesus CD4. Other escape pathways associated with clear fitness costs to the virus. Our data report effective protection of rhesus macaques from SIVmac239.

One sentence summary:

AAV-expressed eCD4-Ig protects rhesus macaques from SIVmac239.

Introduction

Conventional HIV-1 vaccine designs have not been successful due in part to their inability to elicit neutralizing antibodies. However, over the past decade, broadly neutralizing antibodies (bNAbs) that emerge naturally several years post-infection in some HIV-1-positive individuals have been identified and characterized (1). Whereas vaccine immunogen design is currently focused on eliciting such bNAbs, passive administration of bNAbs has successfully prevented and treated SHIV infection in rhesus macaques (2–9). Additionally, clinical trials have shown bNAbs to be safe in uninfected individuals and both safe and effective in HIV-1-infected individuals (10–17).

A limitation to passive administration of bNAbs is the need for repeated infusions to maintain concentrations at protective and therapeutic levels. Adeno-associated virus (AAV) vectors can bypass this difficulty by expressing a secreted protein for years after a single inoculation (18). These vectors can deliver a transgene payload to the nuclei of transduced tissue including skeletal muscle and liver. The transgenes persists as an unintegrated episome that can express a protein of interest. AAV-expressed bNAbs or immunoadhesins can also prevent HIV-1 or SIVmac316 transmission in animal models (19–21). However, more recent macaque studies show that AAV-delivered bNAbs, either of rhesus origin or rhesusized in their constant regions, are frequently targeted by host immune responses, limiting their expression and efficacy (22–24). The macaques often develop severe anti-drug antibody (ADA) responses which have been correlated to the degree of somatic hypermutation of the expressed bNAbs (22). Additionally, a recent study reported 20% of macaques developing ADA against an anti-SIV antibody with lower amounts of somatic hypermutation compared to HIV-1 bNAbs (25).

We have previously described an HIV-1 entry inhibitor, designated eCD4-Ig, that is more self-like than a bNAb (26–28). eCD4-Ig is an Fc-fusion protein composed of domains 1 and 2 of human CD4 fused to an IgG Fc with a sulfated CCR5-mimetic peptide attached to the Fc C-terminus. This inhibitor functions like an antibody, in that it binds to the CD4- and coreceptor-binding sites on the HIV-1 or SIV envelope glycoprotein to prevent infection. eCD4-Ig shows greater neutralization breadth than any bNAb described to date, as it potently neutralizes all HIV-1, HIV-2, and SIV isolates assayed (28). We have previously demonstrated that AAV-delivered rhesus eCD4-Ig (rh-eCD4-Ig^{I39N}) could protect rhesus macaques from SHIV-AD8 challenges that infected all control macaques (28). rh-eCD4-Ig^{I39N} uses rhesus CD4 domains 1 and 2 modified with an asparagine at residue 39 to better emulate the potency of human eCD4-Ig. As previously reported, this human CD4-derived residue improves binding to both HIV-1 and SIV Env proteins (29, 30). In this study, we examined the efficacy of AAV-expressed eCD4-Ig to protect rhesus macaques from SIVmac239 challenges. Taken together with protection observed against SHIV-AD8 challenges, this study highlights the utility of AAV-expressed eCD4-Ig as an alternative to a conventional HIV-1 vaccine.

Results

Complete, long-term eCD4-Ig-mediated protection from SHIV-AD8

We have reported that AAV1-delivered rh-eCD4-Ig^{I39N} protected 4 of 4 rhesus macaques from four infectious challenges with SHIV-AD8, with a final challenge at 34 weeks post-AAV1 inoculation (28). We continued this study through week 60, when rh-eCD4-Ig^{I39N} concentrations for the four macaques were 23, 30, 34, and 52 µg/mL (fig. S1A). As we previously showed, modest ADA responses were observed in two inoculated macaques, but largely disappeared by week 37 and remained below background through week 60 (fig. S1B). All four inoculated animals were challenged again intravenously (IV) at weeks 52 and 56 with doses of SHIV-AD8 that were two- and four-times greater (1600 and 3200 pg p27), respectively, than the highest previous dose (fig. S1C). These challenges were 8- and 16-times the 50% animal infectious dose (AID₅₀) of this stock of SHIV-AD8, but no macaque became infected. In contrast, as we previously reported, AAV1 vectors encoding HIV-1 bNAbs (3BNC117, NIH45–46, 10–1074, or PGT121) (31), did not afford significant protection (p-value = 0.23, Mantel-Cox test) from SHIV-AD8 challenge doses that were 16- and 32-times lower than the final challenge dose of the rh-eCD4-Ig^{I39N} group (fig. S1D). We conclude that following a single inoculation, AAV1-rh-eCD4-Ig^{I39N} can completely protect macaques from high-dose SHIV-AD8 challenges for more than one year.

Robust protection from SIVmac239

To further assess the *in vivo* breadth of AAV1-rh-eCD4-Ig^{I39N}, we subsequently investigated whether rh-eCD4-Ig^{I39N} could protect from the highly pathogenic tier 3 virus SIVmac239. We first confirmed that rh-eCD4-Ig^{I39N} neutralized SIVmac239 produced in HEK293T cells and rhesus macaque peripheral blood mononuclear cells (PBMCs) with nearly identical efficiencies, with 50% inhibitory concentrations (IC₅₀) of 0.03 µg/mL and 0.036 µg/mL, respectively (fig. S2). We then inoculated four macaques with 2.0×10¹³ genome copies (GC) AAV1 vectors encoding rh-eCD4-Ig^{I39N} together with AAV1 vectors encoding rhesus

TPST2 (0.5×10^{13} GC), an enzyme that ensures efficient sulfation of tyrosines present at the rh-eCD4-Ig^{I39N} carboxy terminus. Serum rh-eCD4-Ig^{I39N} concentrations peaked at 13–44 µg/mL, plateauing to between 3 and 18 µg/mL (Fig. 1A).

Compared with our previous SHIV-AD8 study (fig. S1B), we observed higher ADA against rh-eCD4-Ig^{I39N} in all four macaques beginning at week 4 post-inoculation (Fig. 1B). However, ADA in rh-eCD4-Ig^{I39N}-expressing macaques reverted to background by 14 weeks in three of four animals, whereas in one animal with the lowest rh-eCD4-Ig^{I39N} concentration (rh2448), ADA persisted for 30 weeks. Increased ADA was observed in all four animals post-infection, as shown for rh2448. ADA for this group of macaques primarily recognized the rh-eCD4-Ig^{I39N} sulfopeptide (Fig. 2A and B), with little or no recognition of the CD4 domains of rh-eCD4-Ig^{I39N}, or the ectodomains of human or rhesus CD4 (Fig. 2C and D).

Beginning at 22 weeks post-AAV1 inoculation, rh-CD4-Ig^{I39N}-expressing macaques were challenged with SIVmac239. SIVmac239 was selected because its Env is highly divergent from that of SHIV-AD8, used in fig. S1, and because humoral prophylaxis against this tier 3 virus is considered especially challenging. None of the rh-eCD4-Ig^{I39N}-expressing animals became infected after intravenous SIVmac239 challenges that infected all eight untreated control animals (Fig. 3A). Macaque rh2448, with the highest ADA and lowest rh-eCD4-Ig^{I39N} concentrations (3 µg/ml), became infected 34 weeks post-inoculation at twice the challenge dose that infected all control animals. With repeated escalating challenge inoculum doses, the remaining macaques eventually succumbed to infection at 8-, 16-, and 32-times the dose that infected all control animals, at 42, 46, and 50 weeks post-inoculation, respectively. Although the macaque expressing the lowest amounts of rh-eCD4-Ig^{I39N} became infected first, we did not observe a significant correlation (p-value = 0.33 Pearson correlation analysis) between number of challenges needed to infect the macaques and the amount of expressed rh-eCD4-Ig^{I39N} at the time of SIVmac239 acquisition (fig. S3). Despite the absence of a detectable correlation, this protection derived from rh-eCD4-Ig^{I39N} rather than a non-specific consequence of AAV1 inoculation. For example, unlike serum from rh-eCD4-Ig^{I39N}-inoculated macaques, serum from animals inoculated with AAV1 expressing antibodies did not neutralize SIVmac239 (fig. S4A) or mediate antibody-dependent cellular cytotoxicity (ADCC) against this virus (fig. S4B). We then compared viral loads in infected rh-eCD4-Ig^{I39N}-expressing macaques (Fig. 3B). Interestingly, peak and post-peak (weeks 6 through 12 post-infection) viral loads were significantly lower (p-value < 0.05, unpaired t test) in rh-eCD4-Ig^{I39N}-expressing macaques compared to controls (Fig. 3C and D). This difference is especially noteworthy because rh-eCD4-Ig^{I39N}-expressing macaques were challenged with doses of SIVmac239 that were considerably higher than what control animals received. These latter data suggest that rh-eCD4-Ig^{I39N} exerts continuing pressure on the replicating virus, although it may also suggest differences in initial seeding of infected cells. Collectively, our data show that AAV-delivered rh-eCD4-Ig^{I39N} affords substantial and robust protection against acquisition of both SHIV-AD8 and SIVmac239 and suggest a role in partial control of SIVmac239 replication post-acquisition.

Evolution of SIVmac239 Env in the presence of rhesus eCD4-Ig

To examine whether AAV-expressed rh-eCD4-Ig^{I39N} exerted pressure on the replicating virus in macaques that became infected with SIVmac239, we sequenced full length *env* genes from all four rh-eCD4-Ig^{I39N}-expressing macaques and all eight controls. Samples were obtained from macaques expressing rh-eCD4-Ig^{I39N} at the time of necropsy and from control animals 10–12 weeks post infection, after which they were placed on combination antiretroviral therapy (cART) for another study. We observed multiple amino acid changes in Env sequences in the rh-eCD4-Ig^{I39N} group not found in the control group (fig. S5A and B). Some of these mutations may reflect ongoing escape from the rhesus humoral response, whereas others may confer resistance to rh-eCD4-Ig^{I39N}. We therefore characterized those that emerged multiple times in one or more rh-eCD4-Ig^{I39N}-expressing macaque. Of note, *env* genes from two such macaques, rh2448 and r12080, encoded a deletion of G384 in the CD4 binding loop, analogous to residue 367 of HIV-1 (Fig. 4). Sequences obtained from r13058 encoded mutations at residue 382 (glycine to arginine, G382R) and residue 444 (valine to alanine, V444A), in the CD4 binding loop and C4 region, respectively. No changes in the Loop D region of the CD4-binding site were observed in the rh-eCD4-Ig^{I39N} group. Additionally, no mutations in the CD4-binding site were observed in r13023. Furthermore, we did not observe any mutations in the known residues that comprise the coreceptor-binding site on SIVmac239 (fig. S5B). Surprisingly, sequences from rh2448 and r12080 also encoded mutations at residue 180 (lysine to glutamine or glutamic acid, K180Q or K180E) (Fig. 4). This result is interesting because K180 is in the predicted apex region of SIVmac239, a region of HIV-1 Env shown to bind sulfated tyrosines of apex antibodies such as PGT145, PGDM1400, CAP256-VRC26.25, and PG9 (32, 33).

We then sought to determine whether the observed Env mutations altered susceptibility to rh-eCD4-Ig^{I39N}. Specifically, we generated SIVmac239 gp160 expression plasmids encoding the G382R and V444A mutations (G382R/V444A), the K180Q and G384 deletion mutations (K180Q/G384^Δ), the G382R mutation, or G384 deletion mutation (G384^Δ). When tested in neutralization assays, the K180Q/G384^Δ variant mediated a 48-fold increase in IC₅₀ by rh-eCD4-Ig^{I39N} and the G384^Δ variant alone was 10-fold more resistant than wild-type SIVmac239 (Fig. 5A). A similar decrease in potency against the K180Q/G384^Δ and the G384^Δ variants was also observed for rh-CD4-Ig^{I39N} and rh-CD4-Ig, suggesting that the G384^Δ mutation resulted in a loss of rhesus CD4 binding. In contrast, the G382R/V444A variant was not more resistant to rh-eCD4-Ig^{I39N} or rh-CD4-Ig^{I39N}. However this variant was 3-fold less sensitive to rh-CD4-Ig, lacking the I39N mutation in CD4 domain 1. All three inhibitors, rh-eCD4-Ig^{I39N}, rh-CD4-Ig^{I39N}, and rh-CD4-Ig, showed an increase in potency against the G382R single-mutation variant suggesting the V444A was a compensatory mutation. The neutralization results correlated with the affinity of the gp120 variants on rh-CD4-Ig and rh-CD4-Ig^{I39N} as determined by surface plasmon resonance (SPR) (Fig. 5B and fig. S6). The G382R gp120 variant had greater affinity for rh-CD4-Ig, consistent with previous reports (29). Both the K180Q/G384^Δ and the G384^Δ variants exhibited decreased affinity for rh-CD4-Ig and rh-CD4-Ig^{I39N}. The G382R/V444A variant bound rh-CD4-Ig, but not rh-CD4-Ig^{I39N}, less efficiently, consistent with the decrease in potency observed in the neutralization assay. Finally, we investigated SIVmac239 Env variants from macaque r13023. No Env variants from this macaque included CD4-binding

site changes, but a combination of three gp41 mutations (Y690C/R774S/A833T) resulted in roughly a 13-fold loss of neutralization by rh-eCD4-Ig^{I39N} (Fig. 5C). This Env variant was also similarly more resistant to rh-CD4-Ig^{I39N} (Fig. 5D), suggesting that these gp41 changes resulted in less efficient binding to CD4. Two other variants, A131T/I152M and A612T/K643R, did not increase resistance to rh-eCD4-Ig^{I39N}.

Resistance mutations to rh-eCD4-Ig^{I39N} come at a fitness cost

We next determined whether the resistance mutations reported in Figure 5 had an effect on infection of cells expressing rhesus macaque CD4. Interestingly, we observed that the G382R variant was markedly more efficient at infecting Cf2Th cells expressing rhesus CD4 and human CCR5 than was wild-type SIVmac239 (Fig. 6A). This result is consistent with our previous studies showing that G382R, together with H442Y, facilitated binding to and utilization of rhesus CD4 by adapting to rhesus CD4 isoleucine 39 (29). We also observed a modest loss of infection with the G382R/V444A variant and a more pronounced decrease for both the K180Q/G384 and the G384 variants, consistent with their respective abilities to bind rh-CD4-Ig. Infection observed with the gp41 mutations Y690C/R774S/A833T was near background, suggesting a high fitness cost associated with these changes. As shown in Fig 6A, none of these changes conferred CD4 independence to these Envs. To better understand the relationship between the G382R and G382R/V444A mutations in macaque r13058, we analyzed Env sequences from this macaque longitudinally (Fig. 6B). Although the G382R single-mutation variant predominated in weeks 18 through 22 post-infection, the double G382R/V444A variant outcompeted this G382R by week 28, despite the greater infectivity of G382R *in vitro*. Lastly, we sought to confirm these results on rhesus PBMCs (Fig. 6C). As in Cf2Th-rhCD4/CCR5 cells, the G382R variant replicated more efficiently than wild-type SIVmac239 on these cells, whereas the G382R/V444A replicated less efficiently. Thus the late emergence of V444A appears to complement the G382R variant by making it more resistant to rh-eCD4-Ig^{I39N}, but this alteration associates with a fitness cost.

These data of Figs. 5 and 6 indicate that SIVmac239 can resist rh-eCD4-Ig^{I39N} by at least two mechanisms. First, mutations in Env can reduce affinity for the CD4 domain of rh-eCD4-Ig^{I39N}, thereby increasing the IC₅₀ of rh-eCD4-Ig^{I39N}. This is the pathway taken by variants with the CD4-binding site mutation G384 and apparently those with gp41 mutations Y690C/R774S/A833T. This pathway associates with a high fitness cost. Second, Env can exploit a difference between rh-eCD4-Ig^{I39N} and rhesus CD4 at CD4 residue 39 to increase the efficiency with which it binds and utilizes rhesus CD4, as observed with the G382R Env variant. This mutation increased infection of cells expressing rhesus CD4, but also increases sensitivity to rh-eCD4-Ig^{I39N}. A compensatory mutation, V444A, emerged later to increase resistance to rh-eCD4-Ig^{I39N}, and again imposed some cost to viral fitness.

Discussion

In this study we demonstrate the protective efficacy of AAV-delivered rh-eCD4-Ig^{I39N} against SIVmac239 and the *in vivo* adaptation of SIVmac239 to the presence of rh-eCD4-Ig^{I39N}. This study shows complete protection from SIVmac239 at challenge doses that infected all control animals. Additionally, we show that all four macaques expressing rh-

eCD4-Ig^{I39N} for over a year were protected from high doses of SHIV-AD8. These two studies highlight both the *in vivo* breadth of eCD4-Ig and the potential of AAV for HIV-1 prophylaxis. However these studies have several limitations. First, the IV challenge route used in these studies, although a consistent method for infecting macaques, is not the primary route of human infection, and the concentrations eCD4-Ig^{I39N} in the mucosa, the site of most viral transmission events, have not been determined. Also, although eCD4-Ig in these studies was present at concentrations that could potentially neutralize SIVmac239, it is formally possible that protection derives in part from xenoresponses to the AAV1 vectors produced in human cells, as the challenge virus is also grown in human cells, and the control macaques did not receive an analogous vector. However the absence of protection from AAV-expressed neutralizing antibodies (31) where identical xenoresponses would also be expected, argues against any such effect, especially since robust protection was observed with rh-eCD4-Ig^{I39N} using the identical challenge virus. Additionally, samples from macaques expressing rh-eCD4-Ig^{I39N}, and not the control macaques, neutralized SIVmac239 and mediated ADCC activity.

Although rh-eCD4-Ig^{I39N}-expressing macaques resisted doses of SIVmac239 that infected all control animals, these animals were eventually infected at higher doses. We observed that, once infected, these macaques had lower peak and post-peak viral loads, despite having received higher challenge doses. These data suggest that rh-eCD4-Ig^{I39N} exerts pressure on SIVmac239 over at least 10 to 12 weeks after infection (after which all untreated control animals were placed on antiretroviral therapy). Consistent with these observations, we observed in three of four macaques a deletion in a critical region of the CD4-binding site of Env. This G384 mutation frequently paired with a mutation in the Env apex region recognized by sulfotyrosines of tyrosine-sulfated bNAbs (32, 33). This observation raises the interesting possibility that one of the rh-eCD4-Ig^{I39N} sulfopeptides could engage the apex region, either transiently or more stably, before a CD4-induced conformational change disrupts the apex epitope. Interestingly, resistance to the CD4-domain of rh-eCD4-Ig^{I39N} also was observed due to changes in the cytoplasmic tail gp41, consistent with previous reports that show perturbation of the cytoplasmic tail of SIV can increase its neutralization resistance (34, 35).

We further observed a second, unexpected pathway for resistance. Specifically, a CD4-binding site mutation that we have previously shown to accommodate isoleucine 39 of rhesus CD4 (G382R) emerged under pressure from rh-eCD4-Ig^{I39N}. Interestingly, the G382R mutation increased sensitivity to rh-eCD4-Ig^{I39N} while the G382R/V444A remained as resistant as wild-type SIVmac239. The G382R mutation markedly increased the efficiency with which the SIVmac239 Env utilized cell-surface rhesus CD4, accommodating isoleucine 39 present on rhesus CD4 but modified to asparagine in rh-eCD4-Ig^{I39N}. Of course this pathway would not be available to HIV-1 in humans presuming a complete identity in the CD4 domains of human eCD4-Ig and human CD4. Of note, we have yet to identify any Env that fully escapes any human or rhesus eCD4-Ig variant, either in cell culture studies (36), or, as we show here, *in vivo*.

The ability of AAV-rh-eCD4-Ig^{I39N} to protect from SIVmac239 is strongly suggestive that the same approach would be similarly protective in humans. For example, although AAV-

expressed rh-eCD4-Ig^{I39N} is regularly less immunogenic than AAV-expressed rhesusized bNAbs, the ADA activity against eCD4-Ig^{I39N} appeared to attenuate protection in animal rh2448, due to recognition of the sulfopeptide. Modified sulfopeptide variants may be less immunogenic. Also, most adult humans are 10–12-times larger than macaques, and production of AAV vectors remains expensive, so more efficient eCD4-Ig^{I39N} expression and lower production costs will be necessary for broad application of this approach. Finally, the consequences of long-term expression of eCD4-Ig have not been evaluated in humans and a means of halting transgene expression may be important to its use in AAV-mediated prophylaxis.

That said, AAV-eCD4-Ig is one of the most effective vaccine or vaccine alternative thus far tested in primates. Its ability to protect against both SHIV-AD8 and SIVmac239 is unique but wholly consistent with its 100% breadth in cell culture studies. Unlike current conventional HIV-1 vaccines, this approach only requires a one-time inoculation, and protection is clearly durable, lasting more than a year. Some of these properties may also extend to combinations of AAV-expressed bNAbs, where available data suggest the challenges of ADA (22, 31) and population-level escape will nonetheless be greater. In addition, all AAV-based vaccine approaches require further study of their safety. Nonetheless, our data make clear that AAV-eCD4-Ig remains a viable approach to providing sustained, effective, and universal protection from HIV-1.

Materials and Methods

Study Design

The purpose of this study was to determine whether rhesus macaques expressing rh-eCD4-Ig^{I39N} from AAV1 vectors were protected from SIVmac239 challenges. SIVmac239 is a hard-to-neutralize, tier 3 isolate and considered more difficult for vaccine studies than SHIV challenge viruses. Additionally, we sought to characterize Env escape mutations that arose in macaques expressing rh-eCD4-Ig^{I39N} and infected with SIVmac239. Twelve female macaques were age and weight matched and randomly assigned except that the group receiving AAV1-rh-eCD4-Ig^{I39N} were consistently AAV1-seronegative before inoculation, and Mamu A*01- and Mamu A*02-positive animals were distributed equally among inoculated and control groups. Mamu A*01 and Mamu A*02 are two rhesus macaque MHC alleles that have been described to slow SIV-related disease progression. Each group included at least four macaques, a number that provides statistical significance in our previous published study (28). Viral load analyses were blinded. All other animal procedures and experiments were not blinded. The number of experimental replicates are indicated in the figure legends. Primary data is available in data file S1.

Animals, Inoculations, and Challenges

The 20 animals described in this study were Indian-origin rhesus macaques (*Macaca mulatta*), between two- and five years of age at the time of AAV1 inoculations. 8 macaques were housed at the New England Primate Research Center (NEPRC) in accordance with standards set forth by the American Association for Accreditation of Laboratory Animal Care. 12 macaques were housed at the Wisconsin National Primate Research Center

(WNPRC) and they were similarly cared for with standards set forth by the American Association for Accreditation of Laboratory Animal Care and following the guidelines of the Weatherall Report under a protocol approved by the University of Wisconsin Graduate School Animal Care and Use Committee. All studies were performed with the approval of the appropriate Institutional Animal Care and Use Committees (IACUC), protocol numbers 04888 (NEPRC) and G005045 (WNPRC). All macaques were AAV1- and SIV-negative at the beginning of the study. All macaques were negative for Mamu B*08 and B*17 alleles. Macaques that were positive for the Mamu A*01 or A*02 alleles were divided evenly among groups when possible. Macaque weights at the time of AAV inoculation ranged from 2.5 to 6.38 kg. Four macaques were inoculated with 2 mL saline containing 2.0×10^{13} AAV1 genome copies (GC) encoding the rh-eCD4-Ig^{I39N} transgene and 5×10^{12} AAV1 GC encoding the rhesus macaque TPST-2 transgene into both quadriceps (two 0.5 mL per injections per quadriceps muscle). 1 mL of sera was obtained every one to two weeks post-AAV1 inoculation beginning at Week 4. Animals were initially challenged intravenously with 20 pg p27 SIVmac239. Uninfected animals were re-challenged every 4 weeks with twice the previous dose of challenge virus. Animals in the SHIV-AD8 study were challenged with 1600 and 3200 pg p27 SHIV-AD8. Plasma viral loads were quantified by RT-PCR as previously described (37).

Cell Lines and Plasmids

Human embryonic kidney HEK293T cells were obtained from ATCC and grown in DMEM with 10% fetal bovine serum at 37°C. TZM-bl cells were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc, and grown in DMEM with 10% fetal bovine serum at 37°C (38–42). Cf2Th-rhCD4/R5 and Cf2Th-R5 cells were cultured in DMEM with 10% fetal bovine serum, 500 µg/ml G418, and 3 µg/ml puromycin at 37°C as previously described (29, 43). ADCC target and effector cells have been described previously (44) and were a generous gift from Drs. Michael Alpert and David Evans (University of Wisconsin, Madison). Briefly, CEM.NKR-CCR5-sLTR-Luc ADCC target cells, harboring an SIV Tat-inducible luciferase, were derived from CEM.NKR-CCR5 CD4⁺ T Cells obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, contributed by Dr. Alexandra Trkola and have been previously described (44–48). Target cells were grown in R10 media, specifically RPMI supplemented with 10% FBS, 25 mM HEPES, 2 mM L-glutamine, and 0.1 mg/mL Primocin (InvivoGen). The KHYG-1-derived NK cell line expressing rhesus macaque CD16a (FCGR3A allele) have been previously described (44, 45). KHYG-1-derived NK cells were grown in R10 media additionally supplemented with 1 µg/mL cyclosporine, and interleukin-2 (IL-2). The SIVmac239 SpX plasmid was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr. Ronald C. Desrosiers (49). Plasmids encoding rh-eCD4-Ig, rh-CD4-Ig^{I39N}, rh-CD4-Ig, rh-TPST2, CCR5mim2-Ig, and SHIV-AD8-EO have been previously described (28). SIVmac239 gp160, NL4.3 Env, and NL4.3 Env-luc expression plasmids were previously described (29). SIVmac239 gp160 variants were cloned using synthesized gBlocks (IDT) containing the mutations into the SIVmac239 gp160 expression plasmid. SIVmac239 gp120 and variants were cloned by adding in a stop codon at position 526 of the gp160 expression plasmids by round-the-horn site-directed mutagenesis.

Protein production and purification

Production of rh-eCD4-Ig^{I39N} and rh-CD4-Ig variants was performed as previously described (43). Briefly, HEK293T cells in 175 cm² flasks were transfected with 80 µg total DNA/flask at 50% confluency with a calcium phosphate transfection kit (Takara). Plasmids encoding sulfated proteins were cotransfected with a plasmid encoding human tyrosine protein sulfotransferase 2 (TPST2) at a 4:1 ratio. At 12–16 hrs post-transfection, 10% FBS-DMEM media was replaced with serum-free 293 Freestyle media (Invitrogen). Media was collected after 48 hrs, debris was cleared by centrifugation for 10 min at 1,500 g, and supernatant filtered using 0.45 µm filter flasks (Millipore). Proteins were isolated with HiTrap columns (GE Healthcare) and eluted with IgG Elution Buffer (Thermo Scientific) into 1M Tris-HCl Buffer, pH 9.0 (G Biosciences). Buffer was exchanged with PBS and protein concentrated to 1 mg/mL with Amicon Ultra Centrifugation Filters (Millipore). Proteins were stored at 4°C.

SIVmac239 gp120s were produced as previously described (50). Briefly, Expi293F cells were transfected SIVmac239 gp120 or variant expressor plasmids using FectoPro transfection reagent (Polyplus Transfection), per manufacturer's instructions. Supernatants were collected at five days post transfection, filtered with a 0.45 µm filter flask, and mixed with 1 mL Capto Lentil Lectin beads (GE Healthcare) per 200 mL supernatant overnight at 4°C. Supernatants were then run through a column and beads were collected. The beads were washed with 30 mL of 0.65 M NaCl in PBS and then 20 mL of PBS. 6 mL of 1 M methyl- α -D mannopyranoside (in PBS) was added to the beads and incubated at 4°C for 1–2 hours. The eluate was collected and subjected to buffer exchange in PBS using Amicon Ultra Centrifugation Filters (Millipore) to a concentration of 1 mg/mL. gp120 proteins were stored at 4°C until use.

TZM-bl neutralization assay

TZM-bl neutralization assays were performed as previously described (51). Briefly, SIVmac239 was pre-incubated with titrated amounts of inhibitor or serum in DMEM (10% FBS) for 1 hour at 37°C. TZM-bl cells were detached by trypsinization, diluted in DMEM (10% FBS) to 100,000 cells/mL and added to the virus/inhibitor mixture. Cells were then incubated for 44 hours at 37°C. Viral entry was analyzed using Britelite Plus (Perkin Elmer) and luciferase was measured using a Victor X3 plate reader (Perkin Elmer).

Luciferase-based Antibody Dependent Cell Cytotoxicity Assay

ADCC assays were performed as previously described (27). Briefly, CEM.NKR-CCR5-sLTR-Luc target cells were infected by spinoculation with SIVmac239SpX (200 ng p27) 4 days prior to assay. On the day of the assay, infected target cells were mixed with NK effector cells at a 10:1 ratio in the presence of heat-inactivated serum. Cell were mixed with heat-inactivated serum samples or known concentrations of rh-eCD4-Ig^{I39N} and incubated for 8 hours at 37°C. ADCC activity was measured by luciferase using BriteLite Plus (Perkin Elmer) and measured using a Victor X3 plate reader (Perkin Elmer).

AAV production and purification

Production of recombinant AAV, at the University of Massachusetts Medical School Vector Core, has been previously described (52). In short, HEK293T cells were transfected with an AAV vector plasmid, a plasmid encoding AAV2 rep and AAV1 cap, and helper plasmid encoding adenovirus genes. After harvesting lysates of transfected cells, AAV was purified through three sequential cesium chloride centrifugation steps. The vector genome copy number (GC/ml) was assessed by RT-PCR. The integrity of AAV particles was verified by electron microscopy (EM) and the purity of the AAV preparations was verified by silver-stained SDS-PAGE.

AAV neutralization assay

Rhesus macaques were screened for preexisting AAV1 neutralizing antibodies using an AAV1.LacZ neutralizing assay. Specifically, Huh7 cells were added to 96 well plates at 5×10^4 cells per well and incubated overnight at 37°C. The next day, 5×10^6 wild-type adenovirus diluted in unsupplemented DMEM was added to each well. Serum samples from macaques were diluted 1:5, 1:10, and 1:20 in DMEM supplemented with 10% FBS. Serum dilutions were then mixed with AAV1.LacZ vector for 1 hour at 37°C. The mixture of AAV1.LacZ vector and macaque serum were added to the plates of Huh7 cells and incubated for 1 hour at 37°C. 100 μ L of medium was removed from the 96 well plates and replaced with DMEM supplemented with 20% FBS. Plates were incubated overnight at 37°C. One day post infection, 40 μ L of medium was removed from the 96 well plates and cells were washed with 200 μ L PBS. All supernatant was removed and 40 μ L Lysis buffer (Thermo Fisher) was added to the cells. Plates were incubated for 10 minutes and 100 μ L of Substrate + Reaction buffer (Thermo Fisher) was added. Plates were incubated for 1 hour and LacZ production was measured on a luminometer (Clarity. Bio-Tek). Infection was calculated by dividing the signal observed in wells from the serum samples by the amount of signal observed in wells without serum.

Challenge virus and pseudovirus production

SIVmac239 for challenge studies was made in 293T cells plated in 175 cm² flasks and transfected with 80 μ g SIVmac239 SpX DNA/flask by calcium phosphate technique. Pseudoviruses for neutralization studies were produced in 293T cells plated in 175 cm² flasks and transfected with 40 μ g NL4.3 Env-luc expression plasmid and 40 μ g SIVmac239 gp160 expression plasmid DNA/flask using the jetPRIME transfection reagent (Polyplus Transfection). At 12 hours post transfection, medium was replaced with fresh DMEM (10% FBS). Medium was harvested at 48 hours post transfection, debris was cleared by centrifugation for 10 min at 1,500 $\times$ g, and filtered using 0.45 μ m filter flasks (Millipore). Virus stocks were aliquoted and frozen at -80°C. Virus titers were determined by an SIV p27 ELISA kit (ABL) for challenge studies and infections studies of rhesus PBMC. Viral titers were determined by an HIV p24 ELISA kit (ABL) for pseudovirus infection studies.

gp120 and anti-transgene ELISAs

Half-area 96-well Costar Assay Plates (Corning) were coated with 5 μ g/mL gp120 (Immune Tech). Plates were washed with PBS-T (PBS + 0.05% Tween-20) twice and blocked with

5% BSA in PBS for 1 hour at 37°C. Serum samples were serially diluted in 5% BSA in PBS and were added to the plate in duplicate. Standard curves were generated with a starting concentration of 4 µg/mL standard protein in 5% BSA then serially diluted and added to the plate in duplicate. Plates were incubated for 1 hour at 37°C. Samples were washed five times with PBS-T and a horseradish peroxidase secondary antibody was added. To determine rh-eCD4-Ig^{I39N} concentrations, a secondary antibody recognizing human IgG1 (Jackson Immuno Research) was used. Plates were incubated for 1 hour at 37°C and then washed ten times with PBS-T. TMB solution (Fisher) was added and plates were incubated at room temperature until their standard curves developed (typically 5–10 minutes). TMB Stop Solution (Southern Biotech) was then added and absorbance was read at 450 nm by a Victor X3 plate reader (Perkin Elmer). Standard curves were analyzed using GraphPad Prism 6.0 software and used to determine protein titers from sera samples.

Anti-rh-eCD4-Ig^{I39N} antibodies were measured in the same manner except that ELISA plates were coated with 5 µg/mL of various constructs. Serum samples from macaques expressing rh-eCD4-Ig^{I39N} were assayed against rh-eCD4-Ig^{I39N}, rh-CD4-Ig^{I39N}, C-terminal CCR5mim2-Ig (human IgG1 Fc and hinge without CD4 domains), and the ectodomains of human and rhesus macaque CD4 (Immune Tech). Serum samples were diluted 10-fold and blocked in 5% BSA in PBS. Anti-transgene antibodies were detected using anti-kappa and anti-lambda secondary antibodies (Southern Biotech). Plates were incubated for 1 hour at 37°C. TMB solution was added for 15 minutes at room temperature. Stop solution was then added and absorbance at 450 nm was measured as described above.

Full length env sequencing

SIVmac239 gp160 sequences were obtained from infected plasma samples as previously described (3, 53). Viral RNA was isolated from plasma samples using a QIAamp viral RNA kit (Qiagen). Isolated RNA was reverse transcribed to cDNA using SuperScript III reverse transcriptase (Invitrogen). cDNA synthesis was performed using 1× RT buffer, 0.5 mM dNTP (New England BioLabs), 5 mM dithiothreitol (DTT), 40 units RNaseOut (Invitrogen), 200 units of SuperScript III reverse transcriptase, and 0.25 mM of SIVenvR1 antisense primer (5' TGT AAT AAA TCC CTT CCA GTC CCC CC 3'). RNA, SIVenvR1, and dNTPs were heated at 65°C for 5 minutes followed by 1 minute at 4°C. RT Buffer, dNTP, DTT, and RNaseOut were then added and the reaction mixture was incubated for one hour at 50°C followed by 55°C for one hour. The reaction mixture was heat inactivated for 15 minutes at 70°C. 1 µL of RNase H (New England Biolabs) was added and the reaction mixture was incubated for 20 minutes at 37°C. SIVmac239 gp160 sequences were PCR amplified using the Platinum Taq (Invitrogen) with the following mixture conditions: 1× buffer, 2 mM MgCl₂, 0.2 mM dNTP, 0.2 µM of sense and antisense primer, and 0.025 units of Platinum Taq polymerase. For the first round of PCR, the primers used were SIVenvF1 (5' CCT CCC CCT CCA GGA CTA GC 3') and SIVenvR1. The primers used for the second round were SIVenvF2 (5' GTT TCT TTA TAA TAG ACA TGG AGA CAC CCT TGA GGG AGC 3') and SIVenvR2 (5' GTT TCT TAT GAG ACA TGT CTA TTG CCA ATT TGT A 3'). Cycle conditions were 94°C for 2 minutes, then 35 cycles of 94°C for 15 seconds, 55°C for 30 seconds, and 68°C for 4 minutes, and a final extension of 68°C for 10 minutes. 2 µL of the first PCR was used as the template for the second PCR which used the same conditions, but

run for a total of 45 cycles. After the second PCR, 2.5 units of Platinum Taq polymerase were added and the reaction mixture was incubated at 72°C for 15 minutes. The PCR product was gel extracted and eluted with 20 uL of elution buffer (Qiagen). The gel purified PCR product was then cloned into the pcDNA3.1/V5-His vector by Topo TA cloning per manufacturer's instructions. Cloned vectors were then transformed using Top10 competent cells. Sequencing of the SIVmac239 *env* was performed using the following primers: SIVEnvF3 (5' GGT AAT CAT ATC TAT AAT AGA CAT GGA GAC AC 3'), mac239EnvSeqF1 (5' GGG GAA CAA CTC AGT GCC TAC C 3'), mac239EnvSeqF3 (5' GCA CCT CCA GGT TAT GCT TTG C 3'), mac239EnvSeqF5 (5' GCA GAG GAG AGT TCC TCT AC 3'), mac239EnvSeqF7 (5' GCA ACA GCT GTT GGA CGT GG 3'), mac239EnvSeqF9 (5' CCA GCA GAC CCA TAT CCA ACA GG 3'). Sequences were aligned to wild-type SIVmac239 gp160 to identify mutations using the Los Alamos Highlighter tool (https://www.hiv.lanl.gov/content/sequence/HIGHLIGHT/highlighter_top.html).

Surface plasmon resonance

SPR analysis was performed using a Biacore X100, Biacore software, and Biacore reagents (GE Healthcare). A mouse anti-human IgG antibody was immobilized on a CM5 sensor based on the manufacturer's instructions (GE Healthcare). 2 µg/mL of rh-CD4-Ig and rh-CD4-Ig^{I39N} proteins were captured by the anti-human IgG antibody. For affinity analysis, SIVmac239 gp120s were injected at 400 nM, 200 nM, 100 nM, 50 nM, and 25 nM concentrations. Each analysis included an empty flow cell to deduct background signal. Sensor chips were regenerated after each concentration of gp120 using 3M MgCl₂. Association (k_{on}) and dissociation (k_{off}) rate constants were calculated using a 1:1 Langmuir binding model via the Biacore software. The equilibrium dissociation constant (K_D) was calculated from k_{off}/k_{on} .

Infection assays

HIV-1 pseudovirus bearing SIVmac239 variant Envs and encoding firefly luciferase were diluted to 30 ng p24 and serially diluted 3-fold in DMEM (10% FBS). CD4-negative and rhesus CD4-positive Cf2Th-CCR5 cells were harvested and diluted in DMEM (10% FBS) to 100,000 cells/mL and added to the pseudovirus/inhibitor mixture. Cells were then incubated for 48 hours at 37°C. Viral entry was analyzed using Britelite Plus (Perkin Elmer) and luciferase was measured by a Victor X3 plate reader (Perkin Elmer).

Rhesus macaque PBMCs from a single donor (iQ Biosciences) isolated by Ficoll gradient centrifugation were infected with replication-competent SIVmac239 or variants with the G382R or G382R/V444A mutations as previously described (28). Briefly, rhesus PBMCs were thawed and grown for three days in RPMI supplemented with 20% FBS, 100 U/mL penicillin-streptomycin, 10 mM HEPES, and 5 µg/mL of PHA-L. Rhesus PBMCs were then split into a 12-well plate at 1×10^6 cells/mL in RPMI supplemented with 20% FBS, 100 U/mL penicillin-streptomycin, 10 mM HEPES, and 40 U/mL of human IL-2 (growth medium). Cells were infected with 10 ng SIVmac239 or one of the variants in biological triplicates for three hours at 37°C. Cells were pelleted, washed with un-supplemented RPMI, and medium was replaced with fresh growth medium. At days 1, 3, 6, 9, and 12 post

infection, supernatants were collected, cells were pelleted, and replaced with fresh growth medium. Supernatants were analyzed for p27 amounts using an SIV p27 ELISA kit (ABL).

Statistical Analyses

All statistical analyses were performed using GraphPad Prism 6.07 software (GraphPad Software). Comparisons of groups were performed using Student t test (two-tailed) or Mantel-Cox test as reported in the figure legends. A p-value ≤ 0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

The authors would like to thank the staff at the NEPRC and WNPRC for their efforts in handling and performing the required procedures on the animals used in this study, and the staff of the Quantitative Molecular Diagnostics Core of the AIDS and Cancer Virus Program, Frederick National Laboratory for Cancer Research, for expert technical assistance with viral quantitation assays. The authors would also like to thank Dr. Haiyong Peng and Rebecca Goydel for their technical assistance with the Biacore X100.

Funding Support: This project was supported by National Institutes of Health grants R01 AI080324 (M.F.), UM1 AI126623 (M.F., defeatHIV Delaney Cure Collaboratory), P01 AI100263 (G.G., M.F.) and F32AI122980 (M.R.G.), and by the Bill and Melinda Gates Foundation OPP1132169 (M.F.). M.R.G. is a recipient of the NIH Loan Repayment Program award. J.D.L. is supported in part with federal funds from the National Cancer Institute, National Institutes of Health, under contract HHSN261200800001E. Research reported in this publication was supported in part by the Office of the Director, National Institutes of Health under Award Number P51OD011106 to the Wisconsin National Primate Research Center, University of Wisconsin-Madison. This research was conducted in part at a facility constructed with support from Research Facilities Improvement Program grant numbers RR15459-01 and RR020141-01. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services or the National Institutes of Health, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. government.

References and Notes

1. Kwong PD, Mascola JR, HIV-1 Vaccines Based on Antibody Identification, B Cell Ontogeny, and Epitope Structure. *Immunity* 48, 855–871 (2018). [PubMed: 29768174]
2. Gautam R, Nishimura Y, Gaughan N, Gazumyan A, Schoofs T, Buckler-White A, Seaman MS, Swihart BJ, Follmann DA, Nussenzweig MC, Martin MA, A single injection of crystallizable fragment domain–modified antibodies elicits durable protection from SHIV infection. *Nature Medicine* 24, 610–616 (2018).
3. Julg B, Tartaglia LJ, Keele BF, Wagh K, Pegu A, Sok D, Abbink P, Schmidt SD, Wang K, Chen X, Joyce MG, Georgiev IS, Choe M, Kwong PD, Doria-Rose NA, Le K, Louder MK, Bailer RT, Moore PL, Korber B, Seaman MS, Abdool Karim SS, Morris L, Koup RA, Mascola JR, Burton DR, Barouch DH, Broadly neutralizing antibodies targeting the HIV-1 envelope V2 apex confer protection against a clade C SHIV challenge. *Science Translational Medicine* 9, (2017).
4. Julg B, Pegu A, Abbink P, Liu J, Brinkman A, Molloy K, Mojta S, Chandrashekar A, Callow K, Wang K, Chen X, Schmidt SD, Huang J, Koup RA, Seaman MS, Keele BF, Mascola JR, Connors M, Barouch DH, Virological Control by the CD4-Binding Site Antibody N6 in Simian-Human Immunodeficiency Virus-Infected Rhesus Monkeys. *Journal of Virology* 91, e00498–00417 (2017). [PubMed: 28539448]
5. Julg B, Liu P-T, Wagh K, Fischer WM, Abbink P, Mercado NB, Whitney JB, Nkolola JP, McMahan K, Tartaglia LJ, Borducchi EN, Khatiwada S, Kamath M, LeSuer JA, Seaman MS, Schmidt SD, Mascola JR, Burton DR, Korber BT, Barouch DH, Protection against a mixed SHIV challenge by a broadly neutralizing antibody cocktail. *Science Translational Medicine* 9, (2017).

6. Liu J, Ghneim K, Sok D, Bosche WJ, Li Y, Chipriano E, Berkemeier B, Oswald K, Borducchi E, Cabral C, Peter L, Brinkman A, Shetty M, Jimenez J, Mondesir J, Lee B, Giglio P, Chandrashekar A, Abbink P, Colantonio A, Gittens C, Baker C, Wagner W, Lewis MG, Li W, Sekaly R-P, Lifson JD, Burton DR, Barouch DH, Antibody-mediated protection against SHIV challenge includes systemic clearance of distal virus. *Science* 353, 1045–1049 (2016). [PubMed: 27540005]
7. Gautam R, Nishimura Y, Pegu A, Nason MC, Klein F, Gazumyan A, Golijanin J, Buckler-White A, Sadjadpour R, Wang K, Mankoff Z, Schmidt SD, Lifson JD, Mascola JR, Nussenzweig MC, Martin MA, A single injection of anti-HIV-1 antibodies protects against repeated SHIV challenges. *Nature* 533, 105–109 (2016). [PubMed: 27120156]
8. Shingai M, Nishimura Y, Klein F, Mouquet H, Donau OK, Plishka R, Buckler-White A, Seaman M, Piatak M, Lifson JD, Dimitrov D, Nussenzweig MC, Martin MA, Antibody-mediated immunotherapy of macaques chronically infected with SHIV suppresses viraemia. *Nature* 503, 277–280 (2013). [PubMed: 24172896]
9. Barouch DH, Whitney JB, Moldt B, Klein F, Oliveira TY, Liu J, Stephenson KE, Chang H-W, Shekhar K, Gupta S, Nkolola JP, Seaman MS, Smith KM, Borducchi EN, Cabral C, Smith JY, Blackmore S, Sanisetty S, Perry JR, Beck M, Lewis MG, Rinaldi W, Chakraborty AK, Poignard P, Nussenzweig MC, Burton DR, Therapeutic efficacy of potent neutralizing HIV-1-specific monoclonal antibodies in SHIV-infected rhesus monkeys. *Nature* 503, 224–228 (2013). [PubMed: 24172905]
10. Gaudinski MR, Coates EE, Houser KV, Chen GL, Yamshchikov G, Saunders JG, Holman LA, Gordon I, Plummer S, Hendel CS, Conan-Cibotti M, Lorenzo MG, Sitar S, Carlton K, Laurencot C, Bailer RT, Narpala S, McDermott AB, Namboodiri AM, Pandey JP, Schwartz RM, Hu Z, Koup RA, Capparelli E, Graham BS, Mascola JR, Ledgerwood JE, the VRCST, Safety and pharmacokinetics of the Fc-modified HIV-1 human monoclonal antibody VRC01LS: A Phase 1 open-label clinical trial in healthy adults. *PLOS Medicine* 15, e1002493 (2018). [PubMed: 29364886]
11. Mayer KH, Seaton KE, Huang Y, Grunenberg N, Isaacs A, Allen M, Ledgerwood JE, Frank I, Sobieszczyk ME, Baden LR, Rodriguez B, Van Tieu H, Tomaras GD, Deal A, Goodman D, Bailer RT, Ferrari G, Jensen R, Hural J, Graham BS, Mascola JR, Corey L, Montefiori DC, H. P. T. on behalf of the, N. H. I. V. V. T. N. and the, Safety, pharmacokinetics, and immunological activities of multiple intravenous or subcutaneous doses of an anti-HIV monoclonal antibody, VRC01, administered to HIV-uninfected adults: Results of a phase 1 randomized trial. *PLOS Medicine* 14, e1002435 (2017). [PubMed: 29136037]
12. Caskey M, Schoofs T, Gruell H, Settler A, Karagounis T, Kreider EF, Murrell B, Pfeifer N, Nogueira L, Oliveira TY, Learn GH, Cohen YZ, Lehmann C, Gillor D, Shimeliovich I, Unson-O'Brien C, Weiland D, Robles A, Kummerle T, Wyen C, Levin R, Witmer-Pack M, Eren K, Ignacio C, Kiss S, West AP Jr, Mouquet H, Zingman BS, Gulick RM, Keler T, Bjorkman PJ, Seaman MS, Hahn BH, Fatkenheuer G, Schlesinger SJ, Nussenzweig MC, Klein F, Antibody 10–1074 suppresses viremia in HIV-1-infected individuals. *Nat Med* 23, 185–191 (2017). [PubMed: 28092665]
13. Scheid JF, Horwitz JA, Bar-On Y, Kreider EF, Lu C-L, Lorenzi JCC, Feldmann A, Braunschweig M, Nogueira L, Oliveira T, Shimeliovich I, Patel R, Burke L, Cohen YZ, Hadrihan S, Settler A, Witmer-Pack M, West JAP, Juelg B, Keler T, Hawthorne T, Zingman B, Gulick RM, Pfeifer N, Learn GH, Seaman MS, Bjorkman PJ, Klein F, Schlesinger SJ, Walker BD, Hahn BH, Nussenzweig MC, Caskey M, HIV-1 antibody 3BNC117 suppresses viral rebound in humans during treatment interruption. *Nature* 535, 556–560 (2016). [PubMed: 27338952]
14. Bar KJ, Sneller MC, Harrison LJ, Justement JS, Overton ET, Petrone ME, Salantes DB, Seamon CA, Scheinfeld B, Kwan RW, Learn GH, Proschan MA, Kreider EF, Blazkova J, Bardsley M, Refsland EW, Messer M, Clarridge KE, Tustin NB, Madden PJ, Oden K, O'Dell SJ, Jarocki B, Shiakolas AR, Tressler RL, Doria-Rose NA, Bailer RT, Ledgerwood JE, Capparelli EV, Lynch RM, Graham BS, Moir S, Koup RA, Mascola JR, Hoxie JA, Fauci AS, Tebas P, Chun T-W, Effect of HIV Antibody VRC01 on Viral Rebound after Treatment Interruption. *New England Journal of Medicine* 375, 2037–2050 (2016). [PubMed: 27959728]
15. Lynch RM, Boritz E, Coates EE, DeZure A, Madden P, Costner P, Enama ME, Plummer S, Holman L, Hendel CS, Gordon I, Casazza J, Conan-Cibotti M, Migueles SA, Tressler R, Bailer RT, McDermott A, Narpala S, O'Dell S, Wolf G, Lifson JD, Freemire BA, Gorelick RJ, Pandey JP,

Mohan S, Chomont N, Fromentin R, Chun T-W, Fauci AS, Schwartz RM, Koup RA, Douek DC, Hu Z, Capparelli E, Graham BS, Mascola JR, Ledgerwood JE, Virologic effects of broadly neutralizing antibody VRC01 administration during chronic HIV-1 infection. *Science Translational Medicine* 7, 319ra206–319ra206 (2015).

16. Ledgerwood JE, Coates EE, Yamshchikov G, Saunders JG, Holman L, Enama ME, DeZure A, Lynch RM, Gordon I, Plummer S, Hendel CS, Pegu A, Conan-Cibotti M, Sitar S, Bailer RT, Narpala S, McDermott A, Louder M, O'Dell S, Mohan S, Pandey JP, Schwartz RM, Hu Z, Koup RA, Capparelli E, Mascola JR, Graham BS, V. R. C. S. T. the, Safety, pharmacokinetics and neutralization of the broadly neutralizing HIV-1 human monoclonal antibody VRC01 in healthy adults. *Clinical & Experimental Immunology* 182, 289–301 (2015). [PubMed: 26332605]
17. Caskey M, Klein F, Lorenzi JCC, Seaman MS, West AP Jr, Buckley N, Kremer G, Nogueira L, Braunschweig M, Scheid JF, Horwitz JA, Shimeliovich I, Ben-Avraham S, Witmer-Pack M, Platten M, Lehmann C, Burke LA, Hawthorne T, Gorelick RJ, Walker BD, Keler T, Gulick RM, Fatkenheuer G, Schlesinger SJ, Nussenzweig MC, Viraemia suppressed in HIV-1-infected humans by broadly neutralizing antibody 3BNC117. *Nature* 522, 487–491 (2015). [PubMed: 25855300]
18. Buchlis G, Podsakoff GM, Radu A, Hawk SM, Flake AW, Mingozzi F, High KA, Factor IX expression in skeletal muscle of a severe hemophilia B patient 10 years after AAV-mediated gene transfer. *Blood* 119, 3038–3041 (2012). [PubMed: 22271447]
19. Balazs AB, Ouyang Y, Hong CM, Chen J, Nguyen SM, Rao DS, An DS, Baltimore D, Vectored immunoprophylaxis protects humanized mice from mucosal HIV transmission. *Nat Med* 20, 296–300 (2014). [PubMed: 24509526]
20. Balazs AB, Chen J, Hong CM, Rao DS, Yang L, Baltimore D, Antibody-based protection against HIV infection by vectored immunoprophylaxis. *Nature* 481, 81–84 (2012).
21. Johnson PR, Schnepf BC, Zhang J, Connell MJ, Greene SM, Yuste E, Desrosiers RC, Reed Clark K, Vector-mediated gene transfer engenders long-lived neutralizing activity and protection against SIV infection in monkeys. *Nat Med* 15, 901–906 (2009). [PubMed: 19448633]
22. Martinez-Navio JM, Fuchs SP, Pedreño-López S, Rakasz EG, Gao G, Desrosiers RC, Host Anti-antibody Responses Following Adeno-associated Virus-mediated Delivery of Antibodies Against HIV and SIV in Rhesus Monkeys. *Molecular Therapy* 24, 76–86 (2016). [PubMed: 26444083]
23. Saunders KO, Wang L, Joyce MG, Yang Z-Y, Balazs AB, Cheng C, Ko S-Y, Kong W-P, Rudicell RS, Georgiev IS, Duan L, Foulds KE, Donaldson M, Xu L, Schmidt SD, Todd J-P, Baltimore D, Roederer M, Haase AT, Kwong PD, Rao SS, Mascola JR, Nabel GJ, Broadly Neutralizing Human Immunodeficiency Virus Type 1 Antibody Gene Transfer Protects Nonhuman Primates from Mucosal Simian-Human Immunodeficiency Virus Infection. *Journal of Virology* 89, 8334–8345 (2015). [PubMed: 26041300]
24. Fuchs SP, Martinez-Navio JM, Piatak M Jr, Lifson JD, Gao G, Desrosiers RC, AAV-Delivered Antibody Mediates Significant Protective Effects against SIVmac239 Challenge in the Absence of Neutralizing Activity. *PLOS Pathogens* 11, e1005090 (2015). [PubMed: 26248318]
25. Welles HC, Jennewein MF, Mason RD, Narpala S, Wang L, Cheng C, Zhang Y, Todd J-P, Lifson JD, Balazs AB, Alter G, McDermott AB, Mascola JR, Roederer M, Vectored delivery of anti-SIV envelope targeting mAb via AAV8 protects rhesus macaques from repeated limiting dose intrarectal swarm SIVsmE660 challenge. *PLOS Pathogens* 14, e1007395 (2018). [PubMed: 30517201]
26. Fetzter I, Gardner MR, Davis-Gardner ME, Prasad NR, Alfant B, Weber JA, Farzan M, eCD4-Ig Variants That More Potently Neutralize HIV-1. *Journal of Virology* 92, (2018).
27. Davis-Gardner ME, Gardner MR, Alfant B, Farzan M, eCD4-Ig promotes ADCC activity of sera from HIV-1-infected patients. *PLOS Pathogens* 13, e1006786 (2017). [PubMed: 29253851]
28. Gardner MR, Kattenhorn LM, Kondur HR, von Schaewen M, Dorfman T, Chiang JJ, Haworth KG, Decker JM, Alpert MD, Bailey CC, Neale ES, Fellingner CH, Joshi VR, Fuchs SP, Martinez-Navio JM, Quinlan BD, Yao AY, Mouquet H, Gorman J, Zhang B, Poignard P, Nussenzweig MC, Burton DR, Kwong PD, Piatak M, Lifson JD, Gao G, Desrosiers RC, Evans DT, Hahn BH, Ploss A, Cannon PM, Seaman MS, Farzan M, AAV-expressed eCD4-Ig provides durable protection from multiple SHIV challenges. *Nature* 519, 87–91 (2015). [PubMed: 25707797]
29. Fellingner CH, Gardner MR, Bailey CC, Farzan M, SIVmac239, but not SIVmac316, binds and utilizes human CD4 more efficiently than rhesus CD4. *Journal of Virology*, (2017).

30. Humes D, Emery S, Laws E, Overbaugh J, A Species-Specific Amino Acid Difference in the Macaque CD4 Receptor Restricts Replication by Global Circulating HIV-1 Variants Representing Viruses from Recent Infection. *Journal of Virology* 86, 12472–12483 (2012). [PubMed: 22973036]
31. Gardner MR, Fetzer I, Kattenhorn LM, Davis-Gardner ME, Zhou AS, Alfant B, Weber JA, Kondur HR, Martinez-Navio JM, Fuchs SP, Desrosiers RC, Gao G, Lifson JD, Farzan M, Anti-drug Antibody Responses Impair Prophylaxis Mediated by AAV-Delivered HIV-1 Broadly Neutralizing Antibodies. *Molecular Therapy*, (2019).
32. Lee JH, Andrabi R, Su C-Y, Yasmeen A, Julien J-P, Kong L, Wu NC, McBride R, Sok D, Pauthner M, Cottrell CA, Nieusma T, Blattner C, Paulson JC, Klasse PJ, Wilson IA, Burton DR, Ward AB, A Broadly Neutralizing Antibody Targets the Dynamic HIV Envelope Trimer Apex via a Long, Rigidified, and Anionic β -Hairpin Structure. *Immunity* 46, 690–702 (2017). [PubMed: 28423342]
33. Sok D, van Gils MJ, Pauthner M, Julien J-P, Saye-Francisco KL, Hsueh J, Briney B, Lee JH, Le KM, Lee PS, Hua Y, Seaman MS, Moore JP, Ward AB, Wilson IA, Sanders RW, Burton DR, Recombinant HIV envelope trimer selects for quaternary-dependent antibodies targeting the trimer apex. *Proceedings of the National Academy of Sciences* 111, 17624–17629 (2014).
34. White E, Wu F, Chertova E, Bess J, Roser JD, Lifson JD, Hirsch VM, Truncating the gp41 Cytoplasmic Tail of Simian Immunodeficiency Virus Decreases Sensitivity to Neutralizing Antibodies without Increasing the Envelope Content of Virions. *Journal of Virology* 92, (2018).
35. Yuste E, Johnson W, Pavlakis GN, Desrosiers RC, Virion Envelope Content, Infectivity, and Neutralization Sensitivity of Simian Immunodeficiency Virus. *Journal of Virology* 79, 12455–12463 (2005). [PubMed: 16160173]
36. Fellingner CH, Gardner MR, Weber JA, Alfant B, Zhou AS, Farzan M, eCD4-Ig limits HIV-1 escape more effectively than CD4-Ig or a broadly neutralizing antibody. *Journal of Virology*, (2019).
37. Nichole Cline A, Bess JW, Piatak M, Jr JD Lifson, Highly sensitive SIV plasma viral load assay: practical considerations, realistic performance expectations, and application to reverse engineering of vaccines for AIDS. *Journal of Medical Primatology* 34, 303–312 (2005). [PubMed: 16128925]
38. Platt EJ, Bilska M, Kozak SL, Kabat D, Montefiori DC, Evidence that Ecotropic Murine Leukemia Virus Contamination in TZM-bl Cells Does Not Affect the Outcome of Neutralizing Antibody Assays with Human Immunodeficiency Virus Type 1. *Journal of Virology* 83, 8289–8292 (2009). [PubMed: 19474095]
39. Takeuchi Y, McClure MO, Pizzato M, Identification of Gammaretroviruses Constitutively Released from Cell Lines Used for Human Immunodeficiency Virus Research. *Journal of Virology* 82, 12585–12588 (2008). [PubMed: 18842727]
40. Wei X, Decker JM, Liu H, Zhang Z, Arani RB, Kilby JM, Saag MS, Wu X, Shaw GM, Kappes JC, Emergence of Resistant Human Immunodeficiency Virus Type 1 in Patients Receiving Fusion Inhibitor (T-20) Monotherapy. *Antimicrobial Agents and Chemotherapy* 46, 1896–1905 (2002). [PubMed: 12019106]
41. Derdeyn CA, Decker JM, Sfakianos JN, Wu X, O'Brien WA, Ratner L, Kappes JC, Shaw GM, Hunter E, Sensitivity of Human Immunodeficiency Virus Type 1 to the Fusion Inhibitor T-20 Is Modulated by Coreceptor Specificity Defined by the V3 Loop of gp120. *Journal of Virology* 74, 8358–8367 (2000). [PubMed: 10954535]
42. Platt EJ, Wehrly K, Kuhmann SE, Chesebro B, Kabat D, Effects of CCR5 and CD4 Cell Surface Concentrations on Infections by Macrophagetropic Isolates of Human Immunodeficiency Virus Type 1. *Journal of Virology* 72, 2855–2864 (1998). [PubMed: 9525605]
43. Gardner MR, Fellingner CH, Prasad NR, Zhou AS, Kondur HR, Joshi VR, Quinlan BD, Farzan M, CD4-Induced Antibodies Promote Association of the HIV-1 Envelope Glycoprotein with CD4-Binding Site Antibodies. *Journal of Virology* 90, 7822–7832 (2016). [PubMed: 27334589]
44. Alpert MD, Heyer LN, Williams DEJ, Harvey JD, Greenough T, Allhorn M, Evans DT, A Novel Assay for Antibody-Dependent Cell-Mediated Cytotoxicity against HIV-1- or SIV-Infected Cells Reveals Incomplete Overlap with Antibodies Measured by Neutralization and Binding Assays. *Journal of Virology* 86, 12039–12052 (2012). [PubMed: 22933282]
45. Alpert MD, Harvey JD, Lauer WA, Reeves RK, Piatak M Jr., Carville A, Mansfield KG, Lifson JD, Li W, Desrosiers RC, Johnson RP, Evans DT, ADCC Develops Over Time during Persistent

- Infection with Live-Attenuated SIV and Is Associated with Complete Protection against SIVmac251 Challenge. *PLOS Pathogens* 8, e1002890 (2012). [PubMed: 22927823]
46. Trkola A, Matthews J, Gordon C, Ketas T, Moore JP, A Cell Line-Based Neutralization Assay for Primary Human Immunodeficiency Virus Type 1 Isolates That Use either the CCR5 or the CXCR4 Coreceptor. *Journal of Virology* 73, 8966–8974 (1999). [PubMed: 10516002]
 47. Lyerly HK, Reed DL, Matthews TJ, Langlois AJ, Ahearn PA, Stephen J Petteway R, Weinhold KJ, Anti-GP 120 Antibodies from HIV Seropositive Individuals Mediate Broadly Reactive Anti-HIV ADCC. *AIDS Research and Human Retroviruses* 3, 409–422 (1987). [PubMed: 2833917]
 48. Howell DN, Andreotti PE, Dawson JR, Cresswell P, Natural killing target antigens as inducers of interferon: studies with an immunoselected, natural killing-resistant human T lymphoblastoid cell line. *The Journal of Immunology* 134, 971–976 (1985). [PubMed: 3871222]
 49. Regier DA, Desrosiers RC, The complete nucleotide sequence of a pathogenic molecular clone of simian immunodeficiency virus. *AIDS Res Hum Retroviruses* 6, 1221–1231 (1990). [PubMed: 2078405]
 50. Honarmand Ebrahimi K, West GM, Flefil R, Mass spectrometry approach and ELISA reveal the effect of codon optimization on N-linked glycosylation of HIV-1 gp120. *Journal of Proteome Research* 13, 5801–5811 (2014). [PubMed: 25285362]
 51. Li M, Gao F, Mascola JR, Stamatatos L, Polonis VR, Koutsoukos M, Voss G, Goepfert P, Gilbert P, Greene KM, Biliska M, Kothe DL, Salazar-Gonzalez JF, Wei X, Decker JM, Hahn BH, Montefiori DC, Human Immunodeficiency Virus Type 1 env Clones from Acute and Early Subtype B Infections for Standardized Assessments of Vaccine-Elicited Neutralizing Antibodies. *Journal of Virology* 79, 10108–10125 (2005). [PubMed: 16051804]
 52. Mueller C, Ratner D, Zhong L, Esteves-Sena M, Gao G, Production and Discovery of Novel Recombinant Adeno-Associated Viral Vectors. *Current Protocols in Microbiology* 0 14, Unit14D.11–Unit14D.11 (2012).
 53. Buckley KA, Li P-L, Khimani AH, Hofmann-Lehmann R, Liska V, Anderson DC, McClure HM, Ruprecht RM, Convergent evolution of SIV env after independent inoculation of rhesus macaques with infectious proviral DNA. *Virology* 312, 470–480 (2003). [PubMed: 12919751]

Figure 1. rh-eCD4-Ig^{I39N} concentrations and anti-rh-eCD4-Ig^{I39N} responses in macaques inoculated with AAV1-rh-eCD4-Ig^{I39N}.

(A) Serum concentrations of rh-eCD4-Ig^{I39N} in four rhesus macaques inoculated intramuscularly with 2.0×10^{13} genomic copies (GC) of AAV1 vectors encoding rh-eCD4-Ig^{I39N} and 5×10^{12} GC of AAV1 vectors encoding rhesus TPST-2. (B) Anti-rh-eCD4-Ig^{I39N} IgG antibodies in the sera of macaques expressing rh-eCD4-Ig^{I39N}, as determined by ELISA with plates coated with rh-eCD4-Ig^{I39N}. Note that macaque rh2448 (red) became infected at week 36. Error bars represent the range of measured duplicates per experiment. Data are representative of measurements at least two experiments.

Figure 2. Host ADA against expressed rh-cCD4-Ig^{I39N} primarily targets the sulfated CCR5-mimetic peptide.

(A-D) ELISA plates were coated with rh-CD4-Ig^{I39N} (A), CCR5mim2-Ig (B), the human CD4 ectodomain (C), or the rhesus CD4 ectodomain (D). rh-CD4-Ig^{I39N} and CCR5mim2-Ig included human rather than rhesus Fc domains. The CCR5mim2 sulfopeptide of CCR5mim2-Ig is placed at the Fc carboxy-terminus. Values indicate absorbance at 450 nm. Error bars represent the range of duplicates per experiment. Data are representative of measurements of at least two experiments.

Figure 3. AAV1-delivered rh-eCD4-Ig^{I39N} protects rhesus macaques from high-dose challenges with SIVmac239.

(A) Kaplan-Meier curves comparing infection of four rh-eCD4-Ig^{I39N}-expressing (blue) macaques with eight controls (gray). Challenge doses are indicated on the horizontal axis. Significant protection was observed with rh-eCD4-Ig^{I39N}-expressing macaques (p-value = 0.002; Mantel-Cox test). (B) Viral loads of macaques expressing rh-eCD4-Ig^{I39N} (blue) aligned at the time of infection. Gray lines show viral loads of control macaques which were placed on antiviral therapy on week 12. (C-D) Comparison of viral loads peak and post-peak (mean observed after five weeks) between rh-eCD4-Ig^{I39N} (blue) and their corresponding controls (grey). Bars indicate mean and standard deviation. * indicates p-value < 0.05 based on unpaired t test. Viral loads are the result of individual experiments per time point.

SIVmac239	Apex (179-182) KKKE	Loop D (291-298) HGRDNRTI	CD4 binding loop (377-391) NLTAPEGGDPEVTFM	C4 (439-444) NTWHKV
rh2448-1	.E..		-	
rh2448-3	.Q..		-	
rh2448-4	.E..		-	
rh2448-5	.Q..		-	
rh2448-7	.Q..		-	
rh2448-8	.Q..		-	
rh2448-9	.Q..		-	
rh2448-12	.Q..		-	
r12080-2				
r12080-3			-	
r12080-7			-	
r12080-8			-	
r12080-9				
r12080-11			-	
r12080-12			-	
r12080-13				
r12080-16	.E..			
r12080-17	.E..		-	
r13023-2				
r13023-5				
r13023-6				
r13023-7				
r13023-9				
r13023-11				
r13023-12				
r13023-15				
r13023-16				
r13023-19				
r13058-3			R.....	A
r13058-5			R.....	A
r13058-7			R.....	A
r13058-9			R.....	A
r13058-10			R.....	A
r13058-11			R.....	A
r13058-12			R.....	A
r13058-15			R.....	A
r13058-18			R.....	A
r13058-20			R.....	A

Figure 4. Full-length SIVmac239 gp160 sequences from infected macaques expressing rh-eCD4-Ig^{I39N} show unique mutations in the CD4-binding site and apex region.

Distinct apex and CD4-binding site sequences of SIVmac239 cloned from four infected, rh-eCD4-Ig^{I39N}-expressing rhesus macaques. Alignments and numbering are based on wild-type SIVmac239 gp160 sequence (top). Rows indicate individual Env clone from a single macaque. Periods indicate residues with no change. Mutations not found in any of eight infected control macaques are highlighted in red and indicated by the single letter amino acid. Dashes indicate a deletion of residue G384, also unique to rh-eCD4-Ig^{I39N}-expressing macaques.

Figure 5. Two pathways for resistance to rh-eCD4-Ig^{I39N} occur in the CD4-binding site or gp41 region.

(A) Neutralization assays performed with wild-type SIVmac239 Env and the indicated Env variants using the indicated concentration of rh-eCD4-Ig^{I39N} (left), rh-CD4-Ig^{I39N} (middle), or rh-CD4-Ig (right). Neutralization is normalized to SIVmac239 infection alone. (B) SPR analyses showing the association rate constant (k_{on}), dissociation rate constant (k_{off}), and equilibrium dissociation constant (K_d) of SIVmac239 gp120 or gp120 variants to rh-CD4-Ig (left) or rh-CD4-Ig^{I39N} (right). (C-D) Neutralization assays evaluating the indicated mutations from three SIVmac239 Env sequences against rh-eCD4-Ig^{I39N} (C) or Y690C/R774S/A833T against rh-CD4-Ig^{I39N} (D). Error bars in (A, C, and D) indicate standard error of the mean (S.E.M). Neutralization data are representative of at least three experiments.

Figure 6. Fitness costs associated with resistance to rh-eCD4-Ig^{I39N}.

(A) Infection assays of Cf2Th cells stably expressing rh-CD4 and CCR5 (left) or CCR5 alone (right) using the indicated p24 amounts of luciferase-expressing HIV-1 NL4.3 pseudotyped with the indicated SIVmac239 gp160. (B) Percentages of observed Env mutations over the course of infection from macaque r13058 in which the G382R variant was isolated. (C) p27 values of samples harvested from rhesus macaque PBMCs infected ex vivo with indicated SIVmac239 variant. Biological triplicates of rhesus macaque PBMCs were infected with 10 ng of replication-competent SIVmac239 or its variants (G382R or G382R/V444A). Error bars in (A) and (C) indicate range of independent results (n=3). Data are representative of at least two experiments.