

Antibody-based protection against HIV infection by vectored immunoprophylaxis

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Despite tremendous efforts, development of an effective vaccine against human immunodeficiency virus (HIV) has proved an elusive goal. Recently, however, numerous antibodies have been identified that are capable of neutralizing most circulating HIV strains^{1–5}. These antibodies all exhibit an unusually high level of somatic mutation⁶, presumably owing to extensive affinity maturation over the course of continuous exposure to an evolving antigen⁷. Although substantial effort has focused on the design of immunogens capable of eliciting antibodies *de novo* that would target similar epitopes^{8–10}, it remains uncertain whether a conventional vaccine will be able to elicit analogues of the existing broadly neutralizing antibodies. As an alternative to immunization, vector-mediated gene transfer could be used to engineer secretion of the existing broadly neutralizing antibodies into the circulation. Here we describe a practical implementation of this approach, which we call vectored immunoprophylaxis (VIP), which in mice induces lifelong expression of these monoclonal antibodies at high concentrations from a single intramuscular injection. This is achieved using a specialized adeno-associated virus vector optimized for the production of full-length antibody from muscle tissue. We show that humanized mice receiving VIP appear to be fully protected from HIV infection, even when challenged intravenously with very high doses of replication-competent virus. Our results suggest that successful translation of this approach to humans may produce effective prophylaxis against HIV.

Previous efforts to engineer humoral immunity using adeno-associated virus (AAV)-based vectors resulted in modest antibody production¹¹, which was subsequently improved through the use of

alternative capsids¹² and self-complementary AAV (scAAV) vectors¹³ that increase expression at the expense of carrying capacity. Recently, scAAV vectors were used to direct expression of simian immunodeficiency virus (SIV)-neutralizing immunoadhesins consisting of small, artificially fused antibody fragments¹⁴. However, the efficacy of this prophylaxis was limited by an endogenous immune response directed against the immunoadhesin proteins. To ask whether newer capsid serotypes and vector configurations might support long-lived expression of full-length human antibodies from muscle, we produced AAV vectors with the capsid from serotype 8 (ref. 15) that expressed either luciferase or 4E10 HIV neutralizing antibody driven from cytomegalovirus (CMV) promoters and administered them through a single injection of the gastrocnemius muscle (Fig. 1a). Within one week of vector administration, either luciferase or antibody gene expression was detectable (Supplementary Fig. 1a, left and right respectively). Expression continued to rise, achieving maximum levels after 12–16 weeks and then decreasing two- to threefold before stabilizing for the duration of the 64-week study. Given the long-lived nature of this expression, it seemed possible that these vectors could be used to engineer lifelong humoral immunity provided by full-length, fully human antibodies. Hence, we performed a systematic process of vector and transgene optimization to improve the expression characteristics of this system (Supplementary Information). The heavy- and light-chain variable regions of the HIV-neutralizing b12 antibody were cloned into the vector, and AAV stock was produced for intramuscular administration of 1×10^{11} genome copies into the gastrocnemius muscle of two immunodeficient and two immunocompetent mouse strains: NOD/SCID/ γ c (NSG), Rag2/ γ c (RAG), C57BL/6 (B6) and

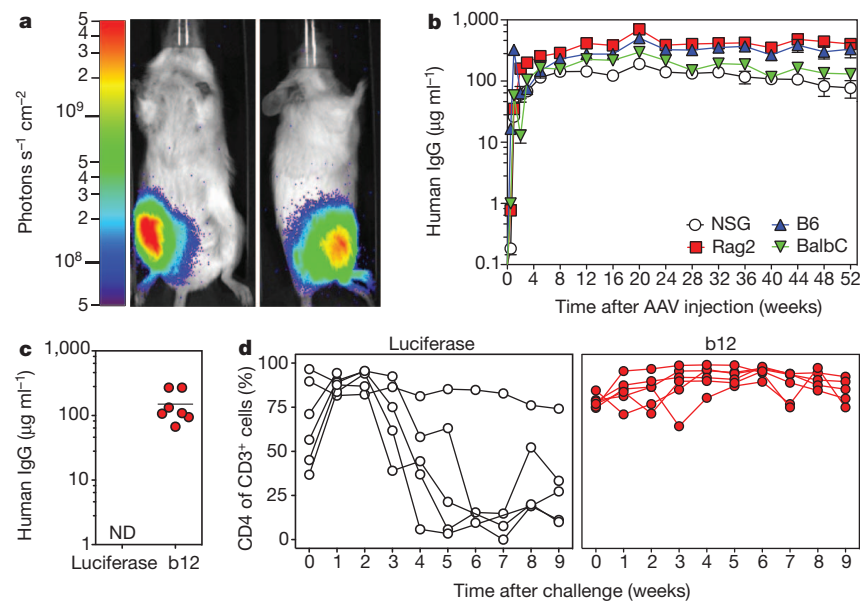


Figure 1 | VIP protects against HIV-mediated CD4 cell depletion in humanized mice.

a, Xenogen imaging of a representative Rag2 mouse 15 weeks after intramuscular injection of 1×10^{10} genome copies of AAV2/8 expressing luciferase. **b**, Quantification of human IgG by ELISA after intramuscular injection of 1×10^{11} genome copies of the optimized expression vector producing b12-IgG in either immunodeficient NOD/SCID/ γ c (NSG) and Rag2/ γ c (Rag2) or immunocompetent C57BL/6 (B6) and Balb/C mice (plot shows mean and standard error, $n = 4$). **c**, Concentration of human IgG in circulation as measured by ELISA on serum samples taken 6 weeks after intramuscular injection of vector expressing either luciferase or b12-IgG (ND, not detected). **d**, Depletion of CD4 T cells in humanized mice after intraperitoneal challenge with 10 ng p24 NL4-3 into animals that received AAV2/8 vectors expressing luciferase (left) or b12-IgG1 (right) 6 weeks earlier ($n = 6$).

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Balb/C. Mice produced the encoded antibody at serum concentrations that were 100-fold higher than the levels achieved with the non-optimized vector, and this level of expression persisted for at least 52 weeks (Fig. 1b compared with Supplementary Fig. 1a, right). In agreement with previous studies of AAV-induced tolerance in mice¹⁶, we detected very limited mouse antibodies raised against human b12-IgG in B6 mice, whereas Balb/C animals generated detectable mouse antibodies against the transgene (data not shown) that did not appear to impact human IgG levels.

To test the ability of VIP to protect mice from challenge *in vivo*, we adapted a previously described humanized mouse model¹⁷ that exhibits CD4 cell depletion following challenge with replication-competent HIV (Supplementary Fig. 5). We administered vector expressing either luciferase or b12 antibody to NSG mice, producing stable serum b12 antibody concentrations of approximately $100 \mu\text{g ml}^{-1}$ within 6 weeks (Fig. 1c). These mice were adoptively populated with expanded human peripheral blood mononuclear cells (huPBMCs), which engrafted over a period of 2 weeks. Mice were then challenged by intraperitoneal injection of the NL4-3 strain of HIV. After HIV challenge, most mice expressing luciferase showed dramatic loss of CD4 cells whereas mice expressing b12 antibody showed no CD4 cell depletion (Fig. 1d).

To compare the protective abilities of the historically available broadly neutralizing antibodies, vectors expressing b12, 2G12, 4E10 and 2F5 were produced and administered to NSG mice. Seven weeks after administration, NSG mice produced $20\text{--}250 \mu\text{g ml}^{-1}$ of the indicated antibodies (Fig. 2a). Interestingly, *in vivo* serum concentrations of 4E10 and 2F5 were somewhat lower than those of b12 and 2G12, despite comparable expression *in vitro* (Supplementary Fig. 3b), perhaps resulting from the previously described self-reactivity of these clones¹⁸. Transduced mice were adoptively populated with huPBMCs, challenged by intravenous injection with HIV and sampled weekly to quantify CD4 cell depletion over time (Fig. 2b). Animals expressing b12 were completely protected from infection, whereas those expressing 2G12, 4E10 and 2F5 were partly protected. Groups demonstrating partial protection consisted of animals with delayed CD4 cell depletion as well as animals that maintained high CD4 cell levels throughout the course of the experiment. Interestingly, mice expressing $250 \mu\text{g ml}^{-1}$ of the 2G12 antibody were only partly protected, despite antibody

levels being over 300-fold higher than previously established half-maximum inhibitory concentration (IC_{50}) values for this antibody-strain combination *in vitro*¹⁹. Eight weeks after challenge, mice were killed and paraffin-embedded spleen sections underwent immunohistochemical staining for the HIV-expressed p24 antigen to quantify the extent of infection (Fig. 2c). Remarkably, mice expressing b12 had no detectable p24-expressing cells, whereas those expressing other antibodies exhibited significant positive staining (Fig. 2d).

To determine the robustness of protection mediated by VIP, a large cohort of mice expressing b12 antibody were adoptively populated with huPBMCs. Before challenge, all mice expressed high levels of human IgG, presumably owing to engrafted human B-cells (Supplementary Fig. 6a), but only those receiving the b12-expressing vector produced IgG specific for gp120, which reached $100 \mu\text{g ml}^{-1}$ (Supplementary Fig. 6b). Mock-infected mice expressing either luciferase or b12 demonstrated consistent high-level CD4 cell engraftment throughout the course of the experiment, showing that transgene toxicity was not contributing to CD4 cell loss (Fig. 3). In contrast, mice expressing luciferase that received 1 ng of HIV experienced rapid and extensive CD4 cell depletion. At higher doses, infection in luciferase-expressing mice became more consistent and resulted in depletion of CD4 cells below the level of detection in some cases (25, 125 ng doses). Remarkably, all mice expressing b12 demonstrated protection from CD4 cell loss, despite receiving HIV doses over 100-fold higher than necessary to deplete seven out of eight control animals (Fig. 3).

As newer anti-HIV antibodies have become available, we have compared the relative efficacy of b12 to VRC01 antibody. VRC01 neutralizes over 90% of circulating HIV strains *in vitro*¹, making it an excellent candidate for human trials. We administered decreasing doses of vector expressing either b12 or VRC01 to NSG mice and monitored expression of the antibodies over time. For both antibodies, we observed clear dose-dependent expression at all time points analysed (Supplementary Fig. 7a and Fig. 4, top). Mice expressing luciferase or antibodies at various levels were adoptively populated with huPBMCs. Just before challenge, a gp120-specific enzyme-linked immunosorbent assay (ELISA) confirmed the effective antibody concentration in each group (Supplementary Fig. 7b and Fig. 4, middle). After intravenous challenge with 10 ng of HIV, CD4 cells were monitored to determine the impact of antibody concentration. An average

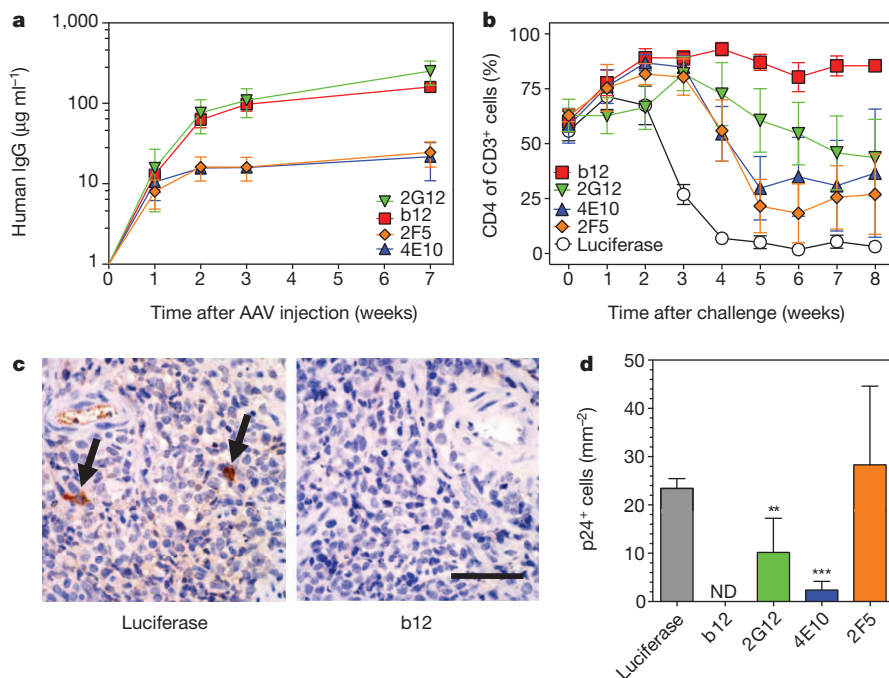


Figure 2 | Comparison of protection mediated by various broadly neutralizing HIV antibodies. **a**, Concentration of antibody in circulation as measured by total human IgG ELISA on serum samples taken after intramuscular injection of vectors expressing four broadly neutralizing HIV antibodies ($n = 8$). **b**, Comparison of the relative effectiveness of four broadly neutralizing HIV antibodies in protecting huPBMC-NSG humanized mice against CD4 cell depletion after intravenous HIV challenge with 5 ng p24 NL4-3 ($n = 8$). **c**, HIV p24 detection by immunohistochemical staining of sections taken from spleens 8 weeks after challenge. Arrows indicate cells scored as positive for p24 expression. Scale bar, 40 μm . **d**, Quantification of immunohistochemical staining of spleen denoting the relative frequency of p24-expressing cells in spleens of infected animals. ND, not detected. Asterisks indicate outcomes significantly different from luciferase control mice versus mice expressing antibodies by two-tailed *t*-test ($n = 4\text{--}6$) ** $P < 0.01$, *** $P < 0.0001$. **a**, **b**, Show mean and s.e.m.; **d** shows mean and s.d.

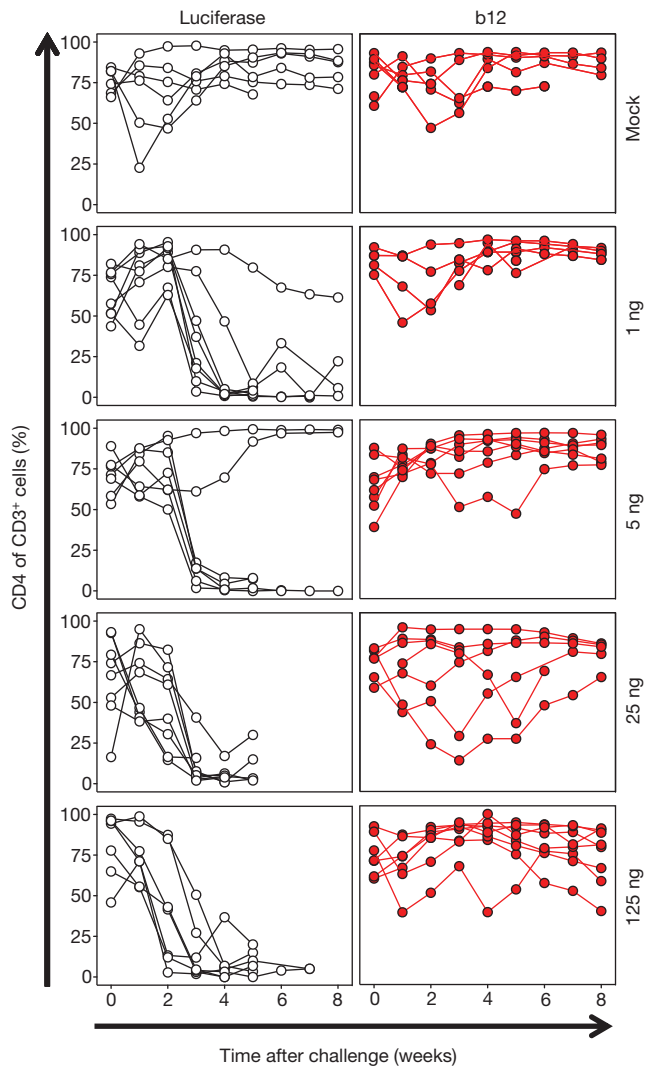


Figure 3 | Robustness of CD4 cell protection mediated by b12 antibody. CD4 cell depletion in huPBMC-NSG humanized mice as a result of intravenous challenge with the dose of NL4-3 indicated on the far right. Mice expressing luciferase (left plots) were susceptible to CD4 cell loss, whereas those expressing b12 (right plots) demonstrated protection from HIV at all doses ($n = 8$).

b12 concentration of $34 \mu\text{g ml}^{-1}$ and VRC01 concentration of $8.3 \mu\text{g ml}^{-1}$ protected mice from infection (Supplementary Fig. 7c and Fig. 4, bottom). Groups expressing lower concentrations of b12 and VRC01 were only partly protected, with several animals showing no detectable loss of CD4 cells and others exhibiting delayed CD4 cell depletion.

Here we demonstrate that broadly neutralizing human antibodies expressed by VIP are capable of protecting animals from even high-dose HIV exposure *in vivo*. Human-to-human HIV transmission rates vary with behaviour but do not generally exceed one per hundred heterosexual exposures²⁰, and recent studies have demonstrated that infections are generally initiated by a single founder strain²¹. Humanized mice with b12 serum concentrations of $100 \mu\text{g ml}^{-1}$ were resistant to HIV infection at challenge doses 100-fold higher than necessary to infect the vast majority of animals, suggesting that this level of protection may far exceed what would be necessary to provide protection against HIV infection in humans. In contrast to previous approaches, VIP produces full-length antibodies that are identical in sequence to those produced by the immune system²². Recent experiments have suggested that full-length antibody structures possess superior *in vitro* neutralization activity compared with modified architectures such as immunoadhesins²³. Use of such naturally occurring antibody architectures should also reduce immune responses against

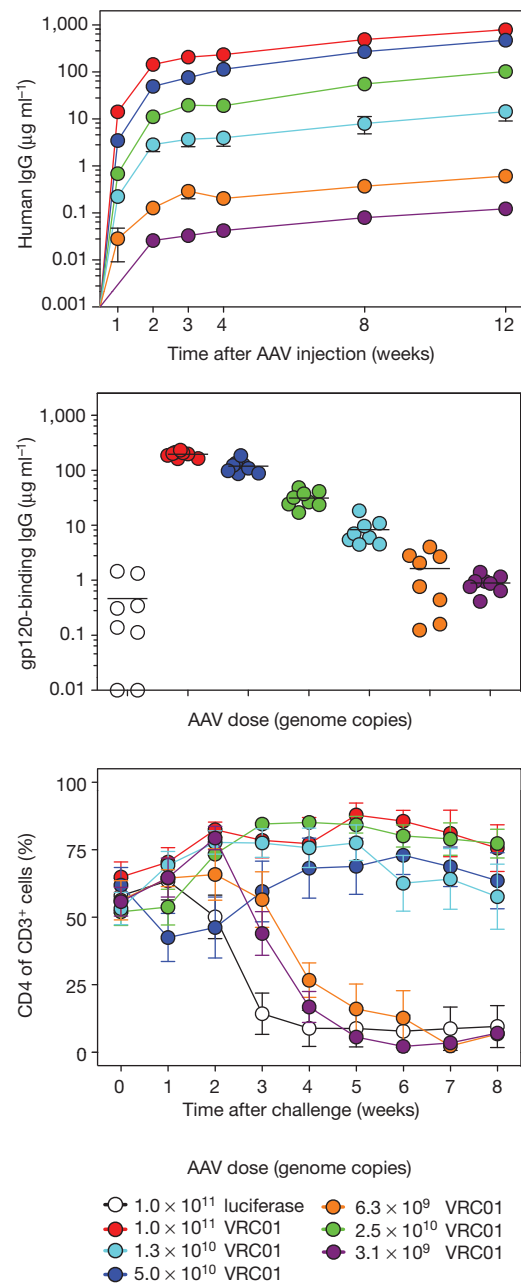


Figure 4 | Determination of the minimum protective dose of VRC01 *in vivo*. Top, VRC01 expression over time as a function of dose as determined by total human IgG ELISA on serum samples taken after AAV administration ($n = 8$). Mice receiving luciferase-expressing vector exhibited no detectable human antibodies ($n = 12$). Middle, concentration of VRC01 in serum 1 day before challenge, 3 weeks after adoptive transfer of human PBMCs and 15 weeks after intramuscular administration of the indicated dose of AAV, as determined by a gp120-specific ELISA to measure the fraction of antibodies capable of binding HIV ($n = 8-12$). Bottom, CD4 cell depletion in huPBMC-NSG humanized mice as a result of intravenous challenge with 10 ng of NL4-3 into animals expressing a range of VRC01, demonstrating the minimum dose of antibody necessary to protect against infection. Top and bottom plots show mean and standard error, middle plot shows individual animals and mean ($n = 8-12$).

the transgene, which were previously shown to reduce the effectiveness of prophylaxis against SIV¹⁴.

Our results demonstrate that VIP administration results in long-lived production of human antibodies at super-prophylactic levels in immunocompetent animals. Clinical trials involving AAV have demonstrated remarkable success when targeting immunoprivileged sites such as retinal tissue²⁴, but transduction of liver resulted in an

adaptive immune response against vector capsid²⁵. Studies in non-human primates have shown that the elicitation of capsid-specific cytotoxic T-lymphocytes is limited to AAV capsids that exhibit heparin-binding activity²⁶. Interestingly, serotypes lacking heparin-binding activity, including AAV8, did not induce CTL responses, suggesting that AAV8-based vectors, like the one we have used, may circumvent previously observed immunological barriers to long-term transduction. Additionally, in contrast to liver transduction, administration of AAV by intramuscular injection has been shown to result in very long-lived, albeit low-level, factor IX expression²⁷, suggesting that the route of administration can significantly impact the duration of expression. Although the expression level attainable in humans remains to be determined, it is worth noting that the significantly longer serum half-life of a human antibody in humans²⁸, as opposed to mice²⁹, may result in higher steady-state levels than those observed in the present study. Regardless of this, our results suggest that even if VIP administration in humans results in serum antibody concentrations 100-fold lower than those observed in mice, it may still confer protection against HIV infection.

Given the urgency that exists in combating the ongoing global HIV pandemic and the incremental progress towards a vaccine, novel modalities of prophylaxis must be explored towards solving this global health crisis. Our work demonstrates the feasibility of directly translating the existing repertoire of broadly neutralizing antibodies into functional immunoprophylaxis with robust protective abilities *in vivo*. As more potent broadly neutralizing HIV antibodies continue to be isolated³⁰, VIP can deliver these in concert with existing antibodies to provide increased potency, broader coverage and greater resistance to escape mutations. This approach may find broad use in the rapid development of effective prophylaxis against any existing or future infectious disease for which broadly neutralizing antibodies can be isolated. Beyond infectious diseases, VIP can be applied to therapeutic regimens in which continuous production of monoclonal antibodies *in vivo* is desirable. Given the level of protection that VIP has demonstrated *in vivo*, we believe that highly effective prophylaxis through expression of existing monoclonal antibodies against HIV in humans is achievable.

METHODS SUMMARY

AAV2/8 was produced by transient transfection and purification from culture supernatant by PEG precipitation and caesium chloride ultracentrifugation. Virus was quantified by quantitative PCR (qPCR) against CMV sequences and functionally validated *in vitro* to confirm gene expression before use *in vivo*. Mice were given single injections with purified vector in the gastrocnemius muscle. Antibody concentration in the serum was determined using an ELISA specific for either total human IgG or human IgG against HIV-gp120. Humanized mice expressing antibodies were produced by adoptive transfer of expanded huPBMCs into mice previously transduced with AAV vectors. HIV challenge was performed by intraperitoneal or intravenous injection, and blood was sampled weekly to determine the ratio of CD4 to CD8 cells by flow cytometry.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions A.B.B. and D.B. conceived the study with assistance from L.Y. A.B.B. designed the experiments. A.B.B., J.C. and C.M.H. performed experiments. A.B.B., J.C. and C.M.H. analysed the data. D.S.R. performed immunohistochemistry and analysis. A.B.B. and D.B. wrote the paper with contributions from all authors.

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METHODS

Construction and cloning of modular AAV transfer vectors. To construct the AAV transfer vector, oligonucleotides encoding the 145-base-pair (bp) AAV2-derived inverted terminal repeat 1 (ITR1) in the 'flip' orientation and ITR2 in the 'flop' orientation flanked by unique restriction sites were synthesized (Integrated DNA Technologies) and annealed before ligation into PBR322 plasmid vector. Subsequently, promoters, transgenes and polyadenylation signals flanked by compatible sites were amplified by PCR and cloned between the ITRs, resulting in a modular AAV transfer vector in which unique combinations of restriction sites flanked each element.

AAV virus production and purification. AAV8 was purified from culture supernatants as described^{31,32} with some modifications. 293T cells were maintained in DMEM medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin mix (Mediatech) and 1% glutamine (Mediatech) in a 5% CO₂ incubator at 37 °C. Three days before transfection, four 15 cm plates were seeded with 3.75×10^6 cells each in 25 ml media. Two hours before transfection, media was changed to 15 ml of fresh media. The AAV backbone vector was co-transfected with helper vectors pHELP (Applied Viromics) and pAAV 2/8 SEED (University of Pennsylvania Vector Core) at a ratio of 0.25:1:2 using BioT transfection reagent (Bioland Scientific). The total amount of DNA used per transfection was 80 µg. Five AAV virus collections were performed at 36, 48, 72, 96 and 120 h after transfection. For each time point, media was filtered through a 0.2 µm filter and 15 ml of fresh media was gently added to the plate. After collection, approximately 75 ml of 5× PEG solution (40% polyethylene glycol, 2.5 M NaCl) was added to the total volume of supernatant collected (~300 ml) and the virus was precipitated on ice for at least 2 h (ref. 33). Precipitated virus was pelleted at 7,277g for 30 min (Sorvall RC 3B Plus, H-6000A rotor) and re-suspended in 1.37 g ml^{-1} caesium chloride. Resuspended virus was split evenly into two Quick-Seal tubes (Beckman) and spun at 329,738g at 20 °C for 24 h (Beckman Coulter, Optima LE-80K, 70Ti rotor). Fractions of 100–200 µl were collected in a 96-well flat-bottom tissue culture plate, and a refractometer was used to quantify the refractive index of 5 µl of each fraction. Wells exhibiting refractive indexes between 1.3755 and 1.3655 were combined and diluted to a final volume of 15 ml using Test Formulation Buffer 2 (TFB2, 100 mM sodium citrate, 10 mM Tris, pH 8)³⁴. Virus was loaded onto 100 kDa MWCO centrifugal filters (Millipore) and subjected to centrifugation at 500g at 4 °C until 1 ml retentate remained. Retained virus was then again diluted to 15 ml total volume in TFB2 and this process was repeated such that the virus was washed three times. Final retentate volume was between 500–1000 µl total, which was aliquoted and stored at –80 °C.

AAV quantification and functional validation. Purified AAV was quantified by qPCR essentially as described³⁵ with the following modifications. Frozen aliquots of AAV were thawed and diluted tenfold in digestion buffer containing 10 units of DNase I (Roche) and incubated at 37 °C for 30 min. DNase-digested virus was serially diluted and 5 µl of each dilution was used in a 15 µl qPCR reaction with Perfecta SYBR Green SuperMix, ROX (Quanta Biosciences) and primers designed against the CMV enhancer (5' CMV: AACGCCAATAGGGACTT TCC and 3' CMV: GGGCGTACTTGGCATATGAT) or the luciferase transgene (5' Luc: ACGTGCAAAGAAGCTACCG and 3' Luc: AATGGGAAGTCACGA AGGTG). Samples were run in duplicate on an Applied Biosystems 7300 Real Time PCR System. The following cycling conditions were used: one cycle of 50 °C for 2 min, one cycle of 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Virus titre was determined by comparison with a standard curve generated using either a purified DNA fragment cut with XhoI/NheI from the pVIP luciferase-expressing vector or a reference standard consisting of purified AAV2/8 expressing 4E10 antibody previously titred against the DNA standard.

To validate the functional activity of each lot of the titred virus, we performed *in vitro* infection assays using 293T cells and measured the concentration of the antibody in the cell supernatant. Twenty-four hours before infection, 12-well plates were seeded with 500K cells in 1 ml of media. Two hours before infection, media was replaced with 500 µl per well of fresh media. Genome copies (10^{11}) of each virus were added to each well and allowed to infect for 6 days. Supernatants were removed and quantified for total IgG production by ELISA.

Mouse strains. Immunodeficient NOD/SCID/ γ c (NSG), immunocompetent C57BL/6 (B6) and Balb/C mice were obtained from the Jackson Laboratory. Immunodeficient Rag2/ γ c mice were obtained from A. Berns.

AAV intramuscular injection and bioluminescent imaging. Aliquots of previously titred viruses were thawed slowly on ice and diluted in TFB2 to achieve the predetermined dose in a 40 µl volume. Mice were anaesthetized by isoflurane inhalation and a single 40 µl injection was administered into the gastrocnemius muscle with a 28G insulin syringe. At various times after vector administration, mice were either bled to determine antibody concentration in serum or imaged using a Xenogen IVIS 200 Series imaging system (Caliper Lifesciences). To image, mice were anaesthetized by isoflurane inhalation and given 100 µl of 15 mg ml^{-1}

D-luciferin (Gold Biotechnology) by intraperitoneal injection. Images were taken between 5 and 10 min after D-luciferin injection.

Quantification of antibody production by ELISA. For detection of total human IgG, ELISA plates were coated with 1 µg per well of goat anti-human IgG-Fc antibody (Bethyl) for 1 h. Plates were blocked with 1% BSA (KPL) in TBS for at least 2 h. Samples were incubated for 1 h at room temperature in TBST containing 1% BSA (KPL), then incubated for 30 min with HRP-conjugated goat anti-human kappa light chain antibody (Bethyl). Sample was detected with TMB Microwell Peroxidase Substrate System (KPL). A standard curve was generated using either Human Reference Serum (Lot 3, Bethyl) or purified Human IgG/Kappa (Bethyl).

For detection of gp120-binding IgG, ELISA plates were coated with 0.04–0.10 µg per well HIV-1 gp120MN protein (Protein Sciences) for 1 h. Plates were blocked with 1% BSA (KPL) in TBS for at least 2 h. Samples were incubated for 1 h at room temperature in TBST containing 1% BSA (KPL), then incubated for 30 min with HRP-conjugated goat anti-human IgG-Fc antibody (Bethyl). Sample was detected with TMB Microwell Peroxidase Substrate System (KPL). A standard curve was generated using either purified b12 or VRC01 protein as appropriate for the samples.

HIV virus production and titring. 293T cells were maintained in DMEM medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin mix (Mediatech), 1% glutamine (Mediatech) in a 5% CO₂ incubator at 37 °C. Three days before transfection, two 15 cm plates were seeded with 3.75×10^6 cells each in 25 ml media. Two hours before transfection, media was changed to 15 ml of new media. Forty micrograms of the pNL4-3 plasmid³⁶ encoding an infectious molecular clone of HIV was transfected using Trans-IT reagent (Mirus) according to the manufacturer's instructions. Supernatant collections were performed at 24, 48 and 72 h after transfection and 15 ml of fresh media was gently added back to plate after each harvest. Pooled supernatants were filtered using a 0.45 µm filter to remove cell debris and aliquoted for storage at –80 °C. HIV was quantified following the manufacturer's instructions using an Alliance HIV-1 p24 antigen ELISA kit (Perkin-Elmer).

***In vitro* HIV protection assay.** *In vitro* neutralization assays in luciferase reporter cells were performed as described³⁷ with the following modifications. TZM-bl cells from the National Institutes of Health AIDS Research and Reference Reagent Program were maintained in DMEM medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin mix (Mediatech), 1% glutamine (Mediatech) in a 5% CO₂ incubator at 37 °C. Before the assays, TZM-bl cells were trypsinized, counted and re-suspended in a concentration of 10^5 cells per millilitre, in a total volume of 15 ml. Cells were mixed with 75 µg ml^{-1} DEAE-dextran and varying concentrations of each antibody as indicated and allowed to incubate on ice during the preparation of the virus. To prepare virus dilutions, stock NL4-3 was diluted to 250 ng ml^{-1} in growth media and subsequently fourfold serially diluted in the assay plate. One hundred microlitres of media containing 10,000 cells pre-incubated with antibody were added to wells containing previously diluted virus. Infection was allowed to proceed for 48 h in a 5% CO₂ incubator at 37 °C. Before reading the plate, 100 µl of BriteLite reagent (Perkin Elmer) was added to each well, and the plate was incubated for 2 min at room temperature. One hundred and twenty microlitres of each well was then transferred to an opaque plate and read by VICTOR3 (Wallac 1420 VICTOR3 plate reader, PerkinElmer).

Production of humanized mice for *in vivo* challenge. Humanized mice were produced essentially as described¹⁷ with the following modifications. Human peripheral mononuclear blood cells (AllCells) were thawed from –80 °C, expanded in RPMI medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin mix (Mediatech), 1% glutamine (Mediatech), 50 µM β-mercaptoethanol, 10 mM HEPES (Gibco), $1 \times$ non-essential amino acids (Gibco), $1 \times$ sodium pyruvate (Gibco) and stimulated for T-cell expansion with 5 µg ml^{-1} phytohemagglutinin (Sigma) and 10 ng ml^{-1} human IL-2 (Peprotech) in a 5% CO₂ incubator at 37 °C. Cells were expanded for 7–13 days before use. For engraftment, 2 million to 4 million cells were injected intraperitoneally into NSG mice in a 300 µl volume of media.

HIV protection experiments. One day before HIV challenge, blood samples from each mouse were subjected both to ELISA for antibody quantification and flow cytometry to determine baseline CD4/CD8 ratios. The following day, mice were challenged through either intraperitoneal or intravenous injection of 100 µl containing the specified dose of HIV diluted in PBS. Infected mice were subjected to weekly blood sampling to determine the ratio of CD4 to CD8 cells in the T-lymphocyte subset by flow cytometry.

Flow cytometry. Blood samples were taken from mice by retro-orbital bleeding and were centrifuged for 5 min at $1,150 \text{ g}$ in a microcentrifuge to separate plasma from cell pellets. Plasma was removed and frozen for future analysis and cell pellets were re-suspended in 1.1 ml of $1 \times$ RBC lysis buffer (Biolegend) and incubated on

ice for at least 10 min to remove red blood cells. After lysis, samples were pelleted at 1,150g in a microcentrifuge for 5 min at room temperature, and stained with 65 μ l of a cocktail containing 5 μ l anti-human CD3-FITC, 5 μ l anti-human CD4-PE, 5 μ l anti-human CD8a-APC antibodies (Biolegend) and 50 μ l of phosphate buffered saline supplemented with 2% fetal bovine serum (PBS+). Samples were washed with 1 ml PBS+ and again pelleted at 1,150g in a microcentrifuge for 5 min. Pelleted cells were re-suspended in 200 μ l of PBS+ supplemented with 2 μ g ml⁻¹ propidium iodide (Invitrogen) and analysed on a FACSCalibur flow cytometer (Beckton-Dickinson). Samples were first gated by CD3 expression before determining the ratio of CD4 to CD8 cells within this subset. Samples containing fewer than 20 CD3⁺ events were excluded from the analysis.

Histological staining for HIV p24. At the conclusion of the *in vivo* challenge experiments, spleens were removed from mice and immersed in 10% neutral buffered formalin for 24 h. After fixation, tissues were removed and placed in 70% ethanol until standard paraffin embedding and processing. Sections (4 μ m thick) were then taken and immunohistochemical staining was performed for HIV-p24 detection using the Kal-1 murine monoclonal antibody and standard antigen retrieval techniques³⁸. The slides were reviewed by a pathologist (D.S.R.)

on an Olympus BX51 light microscope and images obtained using a SPOT Insight Digital Camera (Diagnostic Instruments).

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