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\(^1\). These antibodies all exhibit an unusually high level of somatic mutation\(^2\), presumably owing to extensive affinity maturation over the course of continuous exposure to an evolving antigen. Although substantial effort has focused on the design of immunogens capable of eliciting antibodies \textit{de novo} that would target similar epitopes\(^8\), 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 antigen-specific secretion of full-length antibody from muscle tissue. We show using a specialized adeno-associated virus vector optimized for 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\(\times\)10\(^{11}\) genome copies into the gastrocnemius muscle of two immunodeficient and two immunocompetent mouse strains: NOD/SCID/\(\gamma\)c (NSG), Rag2/\(\gamma\)c (RAG), C57BL/6 (B6) and Balb/C mice. Figure 1 VIP protects against HIV-mediated CD4\(^+\) cell depletion in humanized mice. a, Xenogen imaging of a representative Rag2/\(\gamma\)c mouse 15 weeks after intramuscular injection of 1\(\times\)10\(^{11}\) genome copies of AAV2/8 expressing luciferase. b, Quantification of human IgG by ELISA after intramuscular injection of 1\(\times\)10\(^{11}\) genome copies of the optimized expression vector producing b12-IgG in either immunodeficient NOD/SCID/\(\gamma\)c (NSG) and Rag2/\(\gamma\)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 mice\textsuperscript{15}, 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 model\textsuperscript{17} 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 µg ml\textsuperscript{-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 20–250 µg ml\textsuperscript{-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 clones\textsuperscript{23}. 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 µg ml\textsuperscript{-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\textsubscript{50}) values for this antibody-strain combination in vitro\textsuperscript{14}. 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 adaptively 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 µg ml\textsuperscript{-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\textsuperscript{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 adaptively 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
b12 concentration of 34 μg ml⁻¹ and VRC01 concentration of 8.3 μg 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 exposures⁵, and recent studies have demonstrated that infections are generally initiated by a single founder strain⁶. Humanized mice with b12 serum concentrations of 100 μg 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 system⁷. Recent experiments have suggested that full-length antibody structures possess superior in vitro neutralization activity compared with modified architectures such as immunoadhesins⁸. Use of such naturally occurring antibody architectures should also reduce immune responses against the transgene, which were previously shown to reduce the effectiveness of prophylaxis against SIV⁹.

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 tissue⁶, but transduction of liver resulted in an...
adaptive immune response against vector capsids. Studies in non-human primates have shown that the elicitation of capsid-specific cytotoxic T-lymphocytes is limited to AAV capsids that exhibit hepatic toxicity. 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 expression, 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 humans, as opposed to mice, 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 immunophylaxis with robust protective abilities. As more potent broadly neutralizing HIV antibodies continue to be isolated, 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 Ig 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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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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D-Immunodeficient (Gold Biotechnology) by intraperitoneal injection. Images were taken between 5 and 10 min after D-Immunodeficient 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 TBSB 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 Human Reference Serum (Lot 3, Bethyl) or purified Human IgG/Kappa (Bethyl).

For detection of gp120-binding IgG, ELISA plates were coated with 0.04–0.1 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 TBS 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.

**In vitro HIV protection assay.** In vitro neutralization assays in luciferase reporter cells were performed as described 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. 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 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 7,277 g for 30 min (Sorvall RC 3B Plus, H-6000A rotor) and re-suspended in 1.37 ml g−1 cm−3 aqueous solution. Resuspended virus was split evenly into two Quick-Seal tubes (Beckman) and spun at 329,738 g 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 8.1). Virus was loaded onto 100 kDa MWCO centrifugal filters (Millipore) and subjected to centrifugation at 37°C. Samples were incubated at 4°C for 1 h. After centrifugation, samples were incubated at 37°C for 30 min with HRP-conjugated goat anti-human IgG-Fc antibody (Bethyl). 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).

**Production of humanized mice for in vivo challenge.** Humanized mice were produced essentially as described with the following modifications. Human peripheral mononuclear blood cells (PBMCs) were obtained via density gradient centrifugation and cultured with human hematopoietic cytokines (IL-2, GM-CSF, and IL-7) for 7–10 days before use. For each mouse, 10^7 cells were re-suspended in 1 ml of PBS and injected intraperitoneally. Cells were re-suspended in 1.1 ml of 1× 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 65µl of a cocktail containing 5µl anti-human CD3-FITC, 5µl anti-human CD4-PE, 5µl anti-human CD8a-APC antibodies (Biolegend) and 50µl of phosphate buffered saline supplemented with 2% fetal bovine serum (PBS+). Samples were washed with 1ml PBS+ and again pelleted at 1,150g in a microcentrifuge for 5 min. Pelleted cells were re-suspended in 200µl of PBS+ supplemented with 2µg 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 µm thick) were then taken and immunohistochemical staining was performed for HIV-p24 detection using the Kal-1 murine monoclonal antibody and standard antigen retrieval techniques. 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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