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Vectored immunoprophylaxis protects humanized mice from mucosal HIV transmission

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The vast majority of new HIV infections result from relatively inefficient transmission[1,2] of the virus across mucosal surfaces during sexual intercourse[3]. A consequence of this inefficiency is that small numbers of transmitted founder viruses initiate most heterosexual infections[4]. This natural bottleneck to transmission has stimulated efforts to develop interventions that are aimed at blocking this step of the infection process[5]. Despite the promise of this strategy, clinical trials of preexposure prophylaxis have had limited degrees of success in humans, in part because of lack of adherence to the recommended preexposure treatment regimens[6,7]. In contrast, a number of existing vaccines elicit systemic immunity that protects against mucosal infections, such as the vaccines for influenza[8] and human papilloma virus[9]. We recently demonstrated the ability of vectored immunoprophylaxis (VIP) to prevent intravenous transmission of HIV in humanized mice using broadly neutralizing antibodies[10]. Here we demonstrate that VIP is capable of protecting humanized mice from intravenous as well as vaginal challenge with diverse HIV strains despite repeated exposures. Moreover, animals receiving VIP that expresses a modified VRC07 antibody were completely resistant to repetitive intravaginal challenge by a heterosexually transmitted founder HIV strain[11], suggesting that VIP may be effective in preventing vaginal transmission of HIV between humans.

The description of numerous broadly neutralizing HIV antibodies has invigorated strategies aimed at eliciting similar antibodies in naive patients[12]. In addition, it has been proposed that such antibodies could prevent the transmission of HIV if administered to patients by passive transfer. We and others have described the use of non-integrating adeno-associated virus (AAV) vectors to deliver the genes encoding such antibodies or immunoadhesins to muscle tissues, resulting in their long-term, systemic production. This has led to several demonstrations of the effectiveness of such a strategy to prevent intravenous transmission of simian immunodeficiency virus[13] or laboratory strains of HIV[10]. However, it remains to be shown whether such an approach can be effective against HIV transmission between humans, which typically occurs across mucosal surfaces by HIV strains that are distinct from the laboratory strains currently used to test interventions.

To explore this issue, we determined the ability of VIP to prevent intravenous transmission of both JR-CSF, a CCR5-tropic primary isolate[14], and REJO.c, a CCR5-tropic transmitted molecular founder strain[11]. We administered AAV vectors intramuscularly to establish groups of mice expressing high levels of human b12 IgG, VRC01 IgG or luciferase as a negative control (**Fig. 1a**). After administration and engraftment of human peripheral blood mononuclear cells (PBMCs), we challenged humanized mice intravenously with either JR-CSF or REJO.c (**Fig. 1b**). With either virus challenge we observed robust depletion of $CD4^+$ T cells in animals expressing luciferase. Similarly, all b12-expressing mice challenged with REJO.c exhibited $CD4^+$ T cell depletion, which is consistent with the previously observed resistance of this strain to b12 *in vitro* (**Supplementary Fig. 1**). Moreover, only three of the eight animals expressing b12 exhibited $CD4^+$ T cell protection after JR-CSF challenge (**Fig. 1b**). To determine whether viral escape was responsible for the loss of $CD4^+$ T cells in the remaining animals, we sequenced the viral envelope from mice exhibiting $CD4^+$ T cell depletion and compared these sequences to the known wild-type sequence of JR-CSF (**Fig. 1c**). Notably, envelope sequences obtained from mice expressing the b12 antibody exhibited many of the same common mutations that were present in luciferase-expressing control animals, but the sequences from the b12-expressing mice also contained additional unique mutations at JR-CSF Env residues V372 or M373 (numbered relative to the HXB2 reference strain), both of which have been implicated previously in escape from b12 neutralization[15] (**Fig. 1d**). To determine whether these mutations were responsible for the *in vivo* escape of JR-CSF from b12, we engineered each individual mutation into otherwise wild-type JR-CSF and tested the sensitivity of the resulting viral stocks to either b12 or VRC01 *in vitro* (**Fig. 1e**). We found that either mutation enabled nearly complete resistance to b12, but neither mutation had an effect on VRC01 neutralization. This was true despite both antibodies targeting the CD4 binding site of the viral envelope and likely results from their distinct modes of CD4 binding site recognition[16] (**Supplementary Fig. 2**). When we challenged humanized mice expressing VRC01 with

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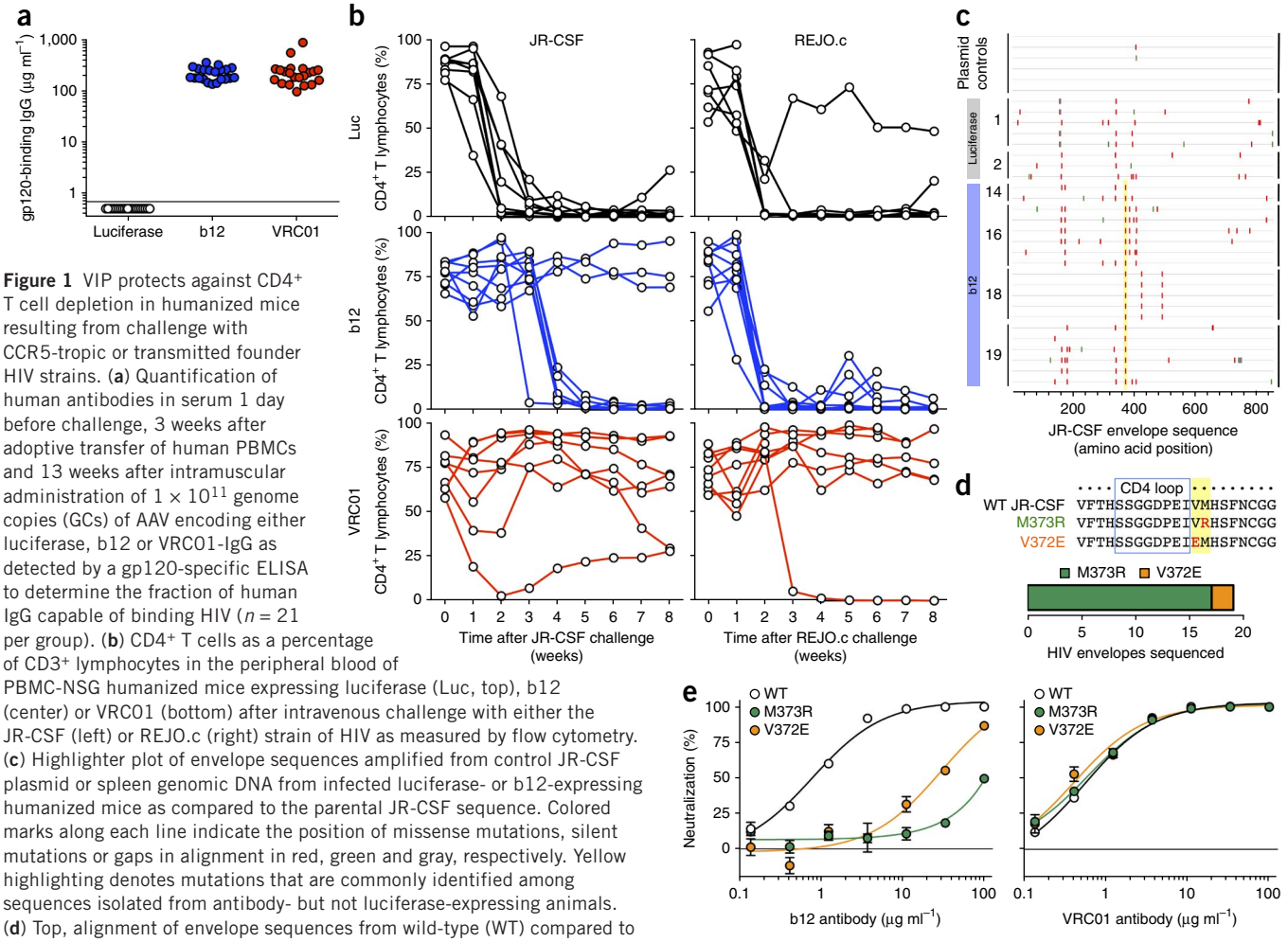

Figure 1 VIP protects against CD4+ T cell depletion in humanized mice resulting from challenge with CCR5-tropic or transmitted founder HIV strains. (**a**) Quantification of human antibodies in serum 1 day before challenge, 3 weeks after adoptive transfer of human PBMCs and 13 weeks after intramuscular administration of 1×10^{11} genome copies (GCs) of AAV encoding either luciferase, b12 or VRC01-IgG as detected by a gp120-specific ELISA to determine the fraction of human IgG capable of binding HIV ($n = 21$ per group). (**b**) CD4+ T cells as a percentage of CD3+ lymphocytes in the peripheral blood of PBMC-NSG humanized mice expressing luciferase (Luc, top), b12 (center) or VRC01 (bottom) after intravenous challenge with either the JR-CSF (left) or REJO.c (right) strain of HIV as measured by flow cytometry. (**c**) Highlighter plot of envelope sequences amplified from control JR-CSF plasmid or spleen genomic DNA from infected luciferase- or b12-expressing humanized mice as compared to the parental JR-CSF sequence. Colored marks along each line indicate the position of missense mutations, silent mutations or gaps in alignment in red, green and gray, respectively. Yellow highlighting denotes mutations that are commonly identified among sequences isolated from antibody- but not luciferase-expressing animals. (**d**) Top, alignment of envelope sequences from wild-type (WT) compared to M373R or V372E mutant JR-CSF demonstrating the location of mutations relative to the CD4 binding loop. Bottom, relative frequency of the two observed escape mutations obtained from four independent b12-expressing mice. (**e**) *In vitro* neutralization assays performed using the TZM-bl cell line infected with either wild-type or the indicated mutant strain of JR-CSF in the presence of serial dilutions of either b12 (left) or VRC01 (right) ($n = 4$ per group). Error bars represent the s.e.m. Each plot contains data generated from one individual experiment, and each experiment was performed once with the indicated number of mice.

JR-CSF or REJO.c, all animals except a single REJO.c-challenged mouse showed at least partial protection of CD4+ T cells (**Fig. 1b**). The single VRC01-expressing mouse that lost all CD4+ T cells exhibited no detectable viral load at any time point tested, and efforts to amplify envelope sequences from either plasma RNA or genomic DNA from this mouse were unsuccessful (data not shown). Consequently, we suspect that this mouse lost CD4+ T cells for reasons unrelated to HIV challenge. Together these results demonstrate that mice can be protected against CCR5-tropic HIV strains by VRC01 but that the b12 monoclonal antibody, which provided robust protection against the CXCR4-tropic NL4-3 strain[10], is easily escaped by the CCR5-tropic JR-CSF strain.

Although these results show that VRC01 is capable of preventing the intravenous transmission of diverse strains, the predominant route of HIV infection worldwide is through heterosexual contact[3]. The bone marrow-liver-thymus (BLT) humanized mouse model, in contrast to nondiabetic severe combined immunodeficient γc (NOD-SCID-γc) humanized mice engrafted with PBMCs (huPBMC-NSG mice), exhibits extensive engraftment of human immune cells into mucosal tissues, which allows for HIV transmission to occur across mucosal surfaces[17]. To create a model of heterosexual transmission that better reflects the stochastic nature of human transmission, we modified the established high-dose, single vaginal challenge model in BLT humanized mice[18] to implement a repetitive, non-abrasive, low-dose viral challenge similar to those used in non-human primates[19]. After administration of VIP encoding VRC01 to BLT mice, we observed production of human IgG specific for HIV gp120 at over 100 μg ml^{-1} in serum, whereas a luciferase-encoding vector produced no specific antibody (**Fig. 2a**). To determine the concentration of antibody reaching the challenge site, we analyzed vaginal wash fluid by ELISA and detected nearly 100 ng ml^{-1} of VRC01 4 weeks after AAV injection and 1 day before the first challenge (**Fig. 2b**). We believe this value to be a minimum estimate of the concentration because of the uncertainty of the dilution resulting from washing a small volume of vaginal mucus. We challenged mice weekly by intravaginal administration of JR-CSF and collected blood samples to monitor CD4+ T cell depletion and viral load. We observed limited but detectable depletion of CD4+ T cells in mice expressing luciferase but a steady or rising number of CD4+ T cells in mice expressing VRC01 (**Fig. 2c**). At the conclusion of the study, we subjected spleens from both groups to immunohistochemistry and observed a substantial number of p24-expressing cells in the spleens of luciferase-expressing control mice,

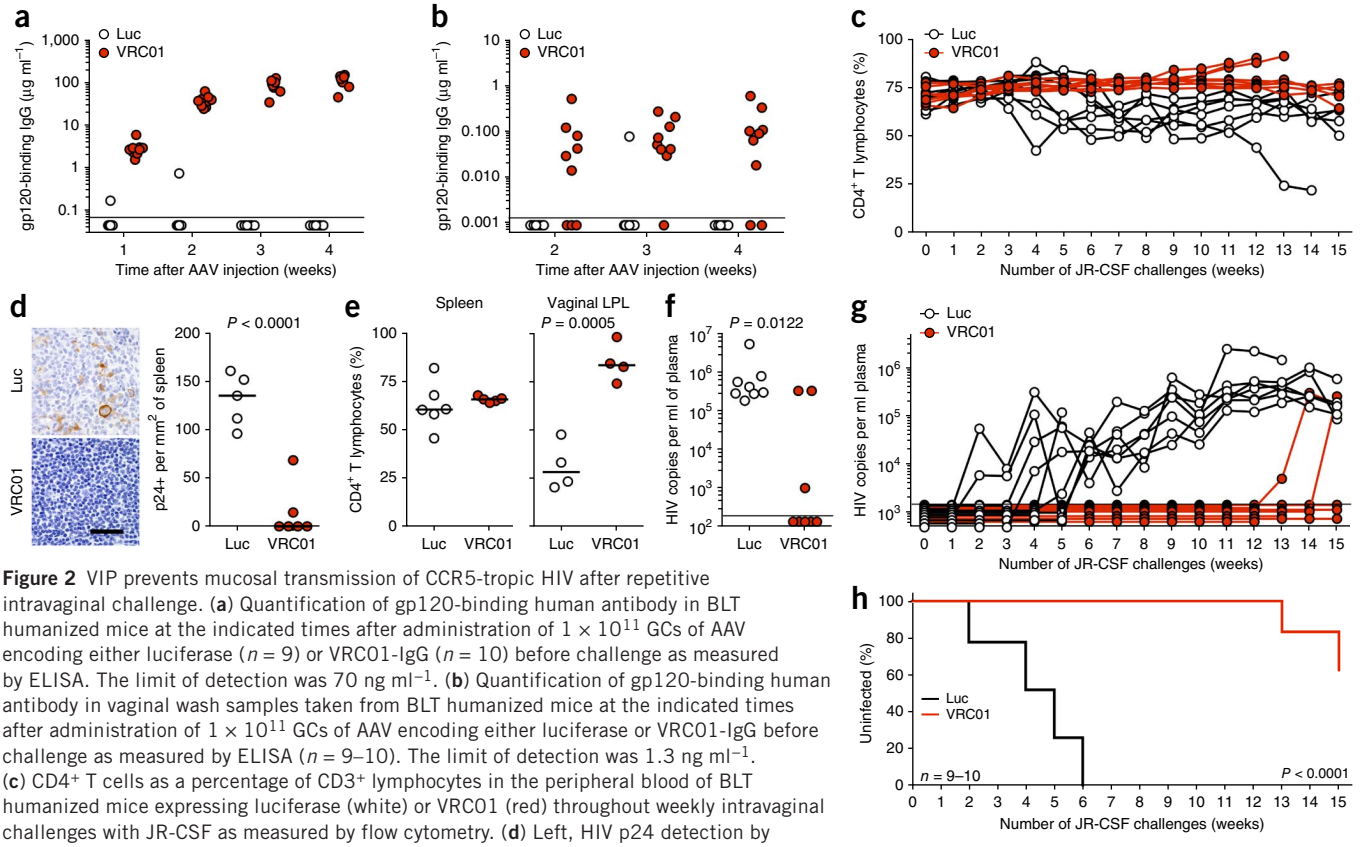

Figure 2 VIP prevents mucosal transmission of CCR5-tropic HIV after repetitive intravaginal challenge. (**a**) Quantification of gp120-binding human antibody in BLT humanized mice at the indicated times after administration of 1×10^{11} GCs of AAV encoding either luciferase ($n = 9$) or VRC01-IgG ($n = 10$) before challenge as measured by ELISA. The limit of detection was 70 ng ml^{-1}. (**b**) Quantification of gp120-binding human antibody in vaginal wash samples taken from BLT humanized mice at the indicated times after administration of 1×10^{11} GCs of AAV encoding either luciferase or VRC01-IgG before challenge as measured by ELISA ($n = 9$–10). The limit of detection was 1.3 ng ml^{-1}. (**c**) CD4$^+$ T cells as a percentage of CD3$^+$ lymphocytes in the peripheral blood of BLT humanized mice expressing luciferase (white) or VRC01 (red) throughout weekly intravaginal challenges with JR-CSF as measured by flow cytometry. (**d**) Left, HIV p24 detection by immunohistochemical (IHC) staining of representative sections taken from spleens of challenged animals. Scale bar, 40 μm. Right, quantification of the IHC staining denoting the relative frequencies of p24-expressing cells in spleens of challenged animals. (**e**) CD4$^+$ T cells as a percentage of CD3-positive T lymphocytes in mouse spleen (left) or vaginal lamina propria (right) tissues after repetitive challenge as detected by flow cytometry. The horizontal lines in **d** and **e** represent the median values. (**f**) HIV viral load detected at the time of euthanasia in the plasma of repetitively challenged BLT mice as detected by the Abbott RealTime HIV-1 Viral Load Assay. The limit of detection was 200 copies ml^{-1}. (**g**) HIV viral load in plasma throughout weekly intravaginal challenge of BLT mice as detected by viral load assay. The limit of detection was 1,500 copies ml^{-1}. (**h**) Fraction of uninfected mice over the course of repetitive intravaginal challenge. Positive infection was defined by two consecutive viral load measurements above the limit of detection ($n = 9$–10). Statistics for grouped comparisons were calculated using either a one- or two-tailed *t* test. Statistics for Kaplan-Meier analyses were calculated by log-rank test. Samples from mice exhibiting fewer than 20 CD3-positive cells were excluded from this analysis. Each plot contains data generated from one individual experiment, and each experiment was performed once with the indicated number of mice.

which were largely absent from mice expressing VRC01 (**Fig. 2d** and **Supplementary Fig. 3**). Although we observed very limited depletion of CD4$^+$ T cells in the spleen by FACS, we found a significant depletion of CD4$^+$ T cells in the vaginal lamina propria of luciferase-expressing mice as compared to VRC01-expressing mice (**Fig. 2e**). Serum samples collected at terminal time points demonstrated that all luciferase-expressing mice were infected, whereas five of eight VRC01-expressing mice exhibited no detectable virus using an ultrasensitive clinical viral load assay (**Fig. 2f**). To determine the time course of infection, we subjected longitudinal serum samples to a viral load assay with reduced sensitivity and observed infection of control mice with a mean of 4.25 ± 1.32 (95% confidence interval (CI)) challenges (**Fig. 2g**). In contrast, this assay indicated that only two VRC01-expressing mice became infected over the duration of the experiment, and this infection occurred only after 13 or 15 exposures, indicating that VIP expressing VRC01 substantially reduced the risk of infection (**Fig. 2h**).

As the available repertoire of broadly neutralizing antibodies against HIV has expanded substantially since our original study[20–22], we set out to determine the minimum protective dose of recently isolated antibodies to ascertain their *in vivo* potency. We gave mice decreasing doses of AAV vectors encoding each antibody or a luciferase-encoding AAV as a control to establish groups of animals expressing a range of antibody concentrations (**Supplementary Fig. 4**). After administration and engraftment of human PBMCs, we challenged mice intravenously with 10 ng p24 of NL4-3 and monitored them weekly for CD4$^+$ T cell decline. We observed protection of humanized mice with a number of antibodies at concentrations as low as 350 ng ml^{-1} (**Table 1** and **Supplementary Fig. 5**). Factoring together the activity we observed and the published breadth of each antibody, we selected the recently described VRC07 antibody containing a G54W mutation[23,24] for further study.

The bottleneck that occurs during mucosal transmission of HIV between humans appears to result in the selection of strains with unique properties that may enhance infectivity[25]. To determine the potential for VRC07 G54W to prevent mucosal transmission in BLT mice, we conducted a second repetitive challenge study with the REJO.c transmitted molecular founder strain of HIV[11]. VRC07 G54W was expressed at concentrations similar to those of VRC01, achieving a concentration of nearly 100 μg ml^{-1} in the serum within 4 weeks of

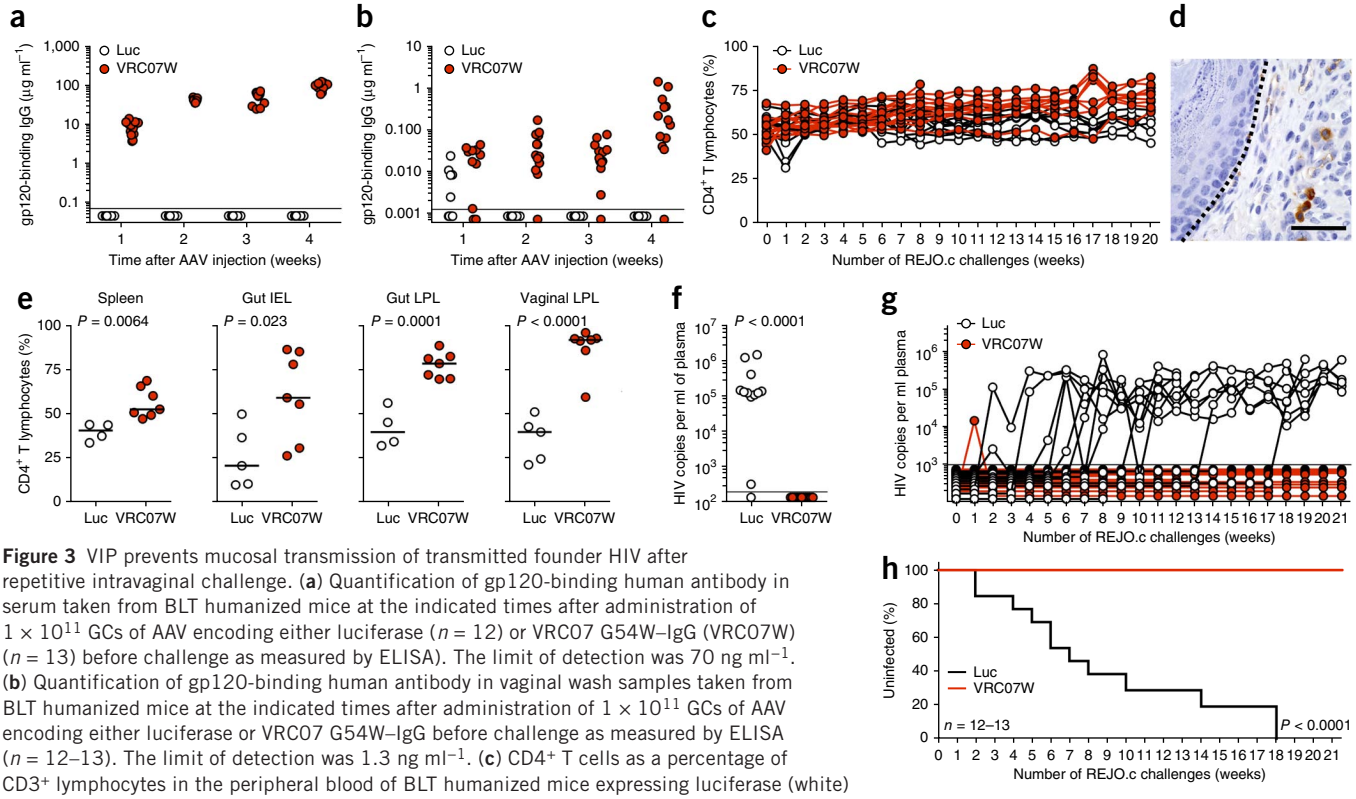

Figure 3 VIP prevents mucosal transmission of transmitted founder HIV after repetitive intravaginal challenge. (**a**) Quantification of gp120-binding human antibody in serum taken from BLT humanized mice at the indicated times after administration of 1×10^{11} GCs of AAV encoding either luciferase ($n = 12$) or VRC07 G54W–IgG (VRC07W) ($n = 13$) before challenge as measured by ELISA). The limit of detection was 70 ng ml^{-1}. (**b**) Quantification of gp120-binding human antibody in vaginal wash samples taken from BLT humanized mice at the indicated times after administration of 1×10^{11} GCs of AAV encoding either luciferase or VRC07 G54W–IgG before challenge as measured by ELISA ($n = 12$–13). The limit of detection was 1.3 ng ml^{-1}. (**c**) CD4+ T cells as a percentage of CD3+ lymphocytes in the peripheral blood of BLT humanized mice expressing luciferase (white) or VRC07 G54W (red) throughout weekly intravaginal challenges with REJO.c as measured by flow cytometry. (**d**) HIV p24 detection by IHC staining of a representative section taken from vaginal tissue of a REJO.c-infected, luciferase-expressing animal. The dashed line represents the interface between the epithelium (left) and the lamina propria (right) showing infected lamina propria lymphocytes. Scale bar, 40 μm. (**e**) CD4+ T cells as a percentage of CD3+ lymphocytes in mouse spleen lymphocytes, gut intraepithelial lymphocytes (IEL), gut lamina propria lymphocytes (LPL) or vagina lamina propria lymphocytes after repetitive challenge of the animals as detected by flow cytometry. The horizontal lines represent the median values. (**f**) HIV viral load at the time of euthanasia in the plasma of repetitively challenged BLT mice as detected by the Abbott RealTime HIV-1 Viral Load Assay. The limit of detection was 200 copies ml^{-1}. (**g**) HIV viral load in the plasma throughout weekly intravaginal challenge of BLT mice as detected by viral load assay. The limit of detection was 1,000 copies ml^{-1}. (**h**) Fraction of uninfected mice over the course of repetitive intravaginal challenge. Positive infection was defined by two consecutive viral load measurements above the limit of detection ($n = 12$–13). Statistics for grouped comparisons were calculated using either a one- or two-tailed *t* test. Statistics for Kaplan-Meier analyses were calculated by log-rank test. Each plot contains data generated from one individual experiment, and each experiment was performed once with the indicated number of mice.

intramuscular injection of the vector (**Fig. 3a**). Notably, we observed levels of VRC07 G54W in the vaginal wash fluid that approached 1 μg ml^{-1} at 4 weeks after AAV injection and 1 day before the first challenge (**Fig. 3b**). After the initiation of weekly intravaginal challenges with REJO.c, we observed that peripheral blood CD4+ T cells were relatively unperturbed in mice expressing luciferase but showed a gradual rise in mice expressing VRC07 G54W (**Fig. 3c**). At the conclusion of the experiment, we analyzed vaginal tissues by immunohistochemistry and observed vaginal lamina propria lymphocytes displaying HIV p24 antigen only in mice expressing luciferase, suggesting that a local infection was taking place at the site of challenge (**Fig. 3d**). FACS analysis of splenic tissue demonstrated a modest but statistically significant reduction in the level of CD4+ T cells (**Fig. 3e**). We observed significant differences among CD4+ T cell populations within gut intraepithelial and lamina propria lymphocytes, as well as in vaginal lamina propria lymphocytes, suggesting that VRC07 G54W was able to protect CD4+ T cells in mucosal tissues, which are typically depleted during the initial phase of HIV infection in patients[26] (**Fig. 3e** and **Supplementary Fig. 6**). In agreement with this observation, the ultrasensitive clinical viral load assay detected infection in nearly all luciferase-expressing control animals, whereas none of the mice producing VRC07 G54W exhibited detectable virus in plasma despite 21 consecutive weekly challenges with REJO.c (**Fig. 3f**). To determine the number of vaginal exposures necessary for infection in this model, we quantified the viral load in longitudinal plasma samples using the less sensitive method described above (**Fig. 3g**). Luciferase-expressing control mice became infected by REJO.c with a mean of 7.45 ± 3.31 (95% CI) challenges. However, in one mouse, just two challenges were sufficient to initiate an infection, whereas in another animal, infection

Table 1 Protection against NL4-3 challenge *in vivo* by the indicated antibody concentration[a]

Vector	Dose administered (GCs)				
	1×10^{11}	5×10^{10}	2.5×10^{10}	1.25×10^{10}	6.25×10^{9}
3BNC117	24.41	7.45	2.43	0.46	0.21
12A12	22.87	7.30	2.59	0.43	0.12
VRC-PG04	31.64	20.49	5.25	1.38	0.35
VRC07	131.42	65.82	26.51	5.76	0.39
VRC07G54W	73.51	35.00	15.30	0.68	0.69
NIH45-46G54W	39.24	13.58	0.87	0.46	0.05
PGT121[b]	256.19	108.51	70.60	13.70	2.89
PGT128[b]	50.34	36.26	49.46	18.97	4.96
PG9	390.61	263.42	134.81	26.32	7.58

Blue indicates protection, yellow indicates partial protection, and red indicates no protection. [a]Antibody concentrations are shown in μg ml^{-1}. [b]These antibodies demonstrated no neutralization activity *in vitro* against the NL4-3 strain.

required 18 challenges. The two control animals that failed to become infected exhibited declining health during the course of the experiment and were euthanized after 8 or 14 challenges. Remarkably, none of the mice expressing VRC07 G54W exhibited a sustained viral load above 1,000 copies ml^{-1} throughout the course of the experiment despite 21 consecutive exposures (**Fig. 3h**).

Taken together, our results suggest that, by providing broadly neutralizing antibodies through VIP, it is possible to protect humanized mice against infection by strains of HIV similar to those that are responsible for human transmission. Notably, we detected viral escape from antibody neutralization despite our use of virus produced from the transfection of molecular clones that would not be expected to result in a diverse virus stock containing many preexisting mutations. We hypothesize that limited viral replication may occur *in vivo*—perhaps locally in the mucosa—despite the presence of neutralizing antibodies, which might allow for selection of resistant strains. Whereas we observed rapid $CD4^+$ T cell decline in huPBMC-NSG control mice challenged with HIV, the kinetics of $CD4^+$ T cell depletion after infection appeared to be substantially slower in BLT control mice. We hypothesize that this difference may be a result of lower levels of xenogenic activation of $CD4^+$ T cells that develop in the BLT model and the regeneration of T cells from engrafted stem cells. Despite the IgG1 isotype expressed by VIP, we found that these antibodies reached the vaginal mucosa. It is unclear whether this mucosal antibody alone was sufficient to prevent transmission or whether protection also required the high concentrations of circulating antibody that were present in our mice. Our results demonstrate that repetitive challenge with a transmitted founder strain can be used to mimic the inefficient nature of vaginal HIV transmission in humans and highlight the utility of such a model as a relatively low-cost approach toward testing new prophylactic interventions. However, it is important to note that in addition to the substantial anatomical differences between mice and humans, the existing BLT humanized mouse model does not entirely recapitulate a functional human immune system. Despite these limitations, it seems reasonable to examine whether a sufficiently high circulating concentration of broadly neutralizing antibody might substantially reduce the probability of sexual transmission of HIV between humans.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

A.B.B. and D.B. conceived the study. A.B.B. designed the experiments. D.S.A. offered suggestions for the experiments and provided the BLT humanized mice. A.B.B., Y.O., C.M.H., J.C. and S.M.N. carried out experiments. A.B.B., Y.O., C.M.H., J.C. and S.M.N. analyzed the data. D.S.R. performed immunohistochemistry and analysis. A.B.B. and D.B. wrote the paper with contributions from all authors.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Statistics, sample size selection and exclusion criteria. All experimental group sizes were chosen to ensure adequate statistical power despite the highly variable nature of the studies performed. No animals were excluded, regardless of their level of human cell engraftment. However, individual samples taken during the course of experiments measured by flow cytometry that exhibited fewer than 20 detectable human $CD3^+$ cells were excluded from analysis to reduce variability. Groups of animals were chosen in consecutive order and were not randomized before commencing the study. Animal studies were not performed in a blinded fashion.

Mouse strains. Immunodeficient male NSG mice were obtained from the Jackson Laboratory at 4–6 weeks of age. BLT mice were immunodeficient female NSG mice obtained from the UCLA breeding colony at 4–6 weeks of age that were transplanted with human fetal liver and thymus tissue under the kidney capsule by the UCLA CFAR humanized mouse core laboratory[27]. All deidentified human tissues utilized for the creation of humanized mice were exempted from Institutional Review Board review. Animal experiments were conducted in compliance with all relevant ethical regulations and were approved by the California Institute of Technology Institutional Animal Care and Use Committee.

AAV virus production and administration. AAV viruses encoding either luciferase or broadly neutralizing monoclonal antibodies were produced as previously described[10]. AAV intramuscular injection was performed as previously described[10]. Briefly, aliquots of previously titered viruses were thawed slowly on ice and diluted to achieve the predetermined dose in a 40 µl volume. A single 40-µl injection was administered into the gastrocnemius muscle of humanized NSG mice with a 28 G insulin syringe. At various times after vector administration, mice were bled to determine antibody concentration in the serum.

Antibody quantification by ELISA. For detection of gp120-binding IgG, ELISA plates were coated with 0.04 µg HIV-1 gp120 MN protein (Protein Sciences) per well overnight at 4 °C. Plates were blocked with 1% BSA (KPL) in Tris-buffered saline (TBS) for 2 h at room temperature. Samples were incubated in TBS plus Tween 20 (TBST) containing 1% BSA (KPL) overnight at 4 °C before incubation with 1:2,500-diluted horseradish peroxidase (HRP)-conjugated goat anti-human IgG-Fc antibody (Bethyl, A80-104A) for 30 min at room temperature. Samples were detected by the TMB Microwell Peroxidase Substrate System (KPL). A standard curve was generated using purified b12, VRC01 or VRC07 G54W protein as appropriate for the samples.

***In vitro* susceptibility assay.** HIV was produced by transient transfection of 293T cells with plasmids encoding NL4-3, JR-CSF or REJO.c (AIDS Reagent program) followed by collection of the supernatant. Supernatants were titered by p24 ELISA (PerkinElmer) to quantify viral concentration. To perform the assay, TZM-bl cells were suspended in medium containing 75 µg ml^{-1} DEAE-dextran at 200,000 cells ml^{-1} and two times the final concentration of either b12 or VRC01 purified antibodies (Caltech Protein Expression Center). After 1 h of incubation at 37 °C, the cell and antibody mixtures were plated and combined with an equal volume of medium containing 27.75 ng p24 of each virus per well (in triplicate) to achieve the final concentration of antibody and allowed to infect overnight. The next day, luciferase expression was determined by adding BriteLite reagent to each well and incubating for 2 min before transferring to an opaque 96-well plate for reading by an automated luminometer (VICTOR).

Intravenous challenge of humanized mice. Humanized NSG mice were produced as previously described[10]. 24 h before intravenous HIV challenge, blood samples were obtained from the mice and subjected to flow cytometry to determine the baseline ratio of CD4 to CD8 and ELISA for antibody quantification. The next day, mice were intravenously challenged with 10 ng p24 of NL4-3, JR-CSF or REJO.c diluted in PBS to a volume of 50 µl. Blood samples of the infected mice were obtained weekly to determine the CD4 to CD8 ratio by flow cytometry.

Flow cytometry. Blood samples were centrifuged at 1,150*g* for 5 min at room temperature to separate the plasma from the cell pellets. Plasma was removed and frozen at −20 °C for future analysis. The cell pellets were resuspended in 1.1 ml of 1× RBC lysis buffer (Biolegend) and incubated on ice for 10 min to remove the red blood cells. After lysis, each sample was pelleted at 1,150*g* in a microcentrifuge for 5 min at room temperature and then stained with 53.75 µl of an antibody cocktail containing 1:43-diluted anti-human CD3-FITC, 1:43-diluted anti-human CD4-phycoerythrin (PE), 1:43-diluted anti-human CD8a-allophycocyanin (APC) (Biolegend, 300406, 300508 and 301014, respectively) and 50 µl of PBS supplemented with 2% FBS (PBS+). Samples were washed once with 1 ml PBS+ and pelleted again at 1,150*g* for 5 min. Pelleted cells were resuspended in 200 µl of PBS+ supplemented with 2 µg ml^{-1} propidium iodide (Invitrogen) and analyzed on MACSQuant flow cytometer (Miltenyi Biotec). 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.

Identification of HIV envelope mutations. Mouse spleens were harvested for genomic DNA isolation. Nested PCR was performed for the *env* gene. The primers used in these reactions were 5′-GCAATAATTGTGTGGTCCATAGTA CTCATAGAATATAGGA and 3′-CCCTATCTGTTGCTGGCTCAGCTCGTC for the first round and 5′-AAAATAGATAGGTTAATTGATAAAATAAGAGA GAGAGCAGAAGACAG and 3′-TCATTCTTTCCCTTACAGTAGACCATC CAGGC for the second round, targeting the *env* gene of HIV JR-CSF strain. For the first-round PCR reaction, 100–300 ng of genomic DNA was used as the template for amplification by KOD Xtreme Hot Start DNA polymerase (EMD) using the following cycling conditions: 1 cycle at 95 °C for 2 min and 20 cycles of 98 °C for 10 s, 65 °C for 30 s and 70 °C for 3 min. For the second-round PCR reaction, 1 µl of the first-round PCR product was used as template for reamplification by KOD Hot Start Master mix (EMD) using the following cycling conditions: 1 cycle at 94 °C for 2 min and 30 cycles of 98 °C for 5 s and 68 °C for 3 min and 30 s. The second-round PCR product was purified by agarose gel electrophoresis and gel extraction (Bioland), and the product was cloned by homologous recombination into an appropriate JR-CSF parental backbone vector using the In-Fusion HD Cloning kit (Clontech). The product was transformed into DH5α competent cells, and positive clones were selected for standard sequencing. Envelope sequences were analyzed using the Los Alamos Highlighter tool (http://www.hiv.lanl.gov/content/sequence/HIGHLIGHT/HIGHLIGHT_XYPLOT/highlighter.html) and numbered relative to HXB2.

Construction of point-directed envelope mutants. Individual mutations selected for further study were introduced into the parental vector expressing the molecular clone using overlapping PCR with primers incorporating the desired change. After amplification of the *env* gene with the primers described above using KOD Hot Start Master mix, the PCR product was purified by gel extraction (Bioland) and cloned by homologous recombination into the appropriate recipient parental backbone vector using the In-Fusion HD Cloning kit (Clontech). The ligation product was transformed into DH5α competent cells, and positive clones were selected for standard sequencing to confirm successful introduction of the desired mutation.

***In vitro* neutralization assay.** To compare the sensitivity of point-mutant viruses to b12 and VRC01 antibody neutralization, we produced each mutant by transient transfection of 293T cells, and viruses in the supernatants were titered by 50% tissue culture infective dose ($TCID_{50}$) assay in TZM-bl cells. Neutralization assays were performed by mixing 250 $TCID_{50}$ of HIV virus in 50 µl with threefold serial dilutions of each antibody and incubation at 37 °C for 1 h. After the incubation, 10,000 TZM-bl cells with 75 µg ml^{-1} of DEAE-dextran (Sigma) were added to each well and incubated at 37 °C for 48 h. Cells were then lysed using BriteLite plus (PerkinElmer) and assayed for luciferase expression using a VICTOR3 luminometer (PerkinElmer). Percentage neutralization was determined by calculating the difference in luminescence between test wells (cells with virus and antibody) and cell control wells (cells only) and dividing this value by the difference between the virus control wells (cells with virus) and the cell control wells.

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Repetitive mucosal challenge experiments. After AAV administration to BLT mice, blood samples and vaginal washes were obtained weekly by retro-orbital bleeding and rinsing the vaginal vault with 20 µl of PBS. 4 weeks after AAV administration, the BLT mice were challenged with 50 ng p24 of JR-CSF or 16 ng p24 of REJO.c in a volume of 20 µl. Virus challenge was performed non-abrasively by placing isoflurane-anesthetized mice in a supine position and elevating the posterior of the animal before shallow insertion of the pipette loaded with virus into the vaginal vault. Virus was administered slowly, and after removal of the pipette, mice were maintained in a supine position for 5 min to prevent loss of the virus. This vaginal challenge protocol was repeated weekly until the conclusion of the experiment. Mice were bled before vaginal challenge each week to obtain samples for $CD4^+$ T cell determination and viral load.

IEL and LPL isolation. After harvesting the spleen, gut and genital tract from BLT mice, fecal material and mucus were removed from the mouse gut and female genital tract by gentle scraping with forceps, and tissues were cut into 0.5-cm fragments and rinsed with cold PBS. Cleaned gut and genital pieces were incubated twice in freshly prepared cell dissociation solution (Ca^{2+} and Mg^{2+} free 1× HBSS, 5 mM EDTA and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)) for 20 min at 37 °C with slow rotation at 100 r.p.m. This fraction was filtered through a 70-µm cell strainer (BD) and considered to contain the IELs. The remainder of the tissues were then incubated twice in freshly prepared LPL isolation solution (25 ml C10 medium with 0.5 mg ml^{-1} type II collagenase for the gut or 12.5 ml C10 medium with 0.5 mg ml^{-1} type II collagenase, 0.1 mg ml^{-1} type I DNase, 25 mM HEPES and 5 mM β-mercaptoethanol for the genital tract) for 30 min at 37 °C with slow rotation at 100 r.p.m. After each incubation, tissues were vortexed for 30 s and drained onto clean metal mesh screens over clean 10-cm dishes to collect supernatants. This fraction was filtered through a 40-µm cell strainer (BD) and considered to contain the LPLs. Cells were further purified by suspension in 10 ml 40% Percoll solution (13.2 ml 100% Percoll with 19.8 ml D10 medium) before underlay of 5 ml of 80% Percoll (13.2 ml 100% Percoll with 3.3 ml 1× PBS), and these gradients were centrifuged at 2,500 r.p.m. for 20 min at 20 °C. Cells at the interface were washed in cold PBS before antibody staining for flow cytometry.

Viral load test by quantitative RT-PCR (qRT-PCR). Viral RNA was extracted using the QIAamp viral RNA mini kit (Qiagen). Each RNA sample was treated with 0.04 U of heparinase I (Sigma-Aldrich) at room temperature for 40 min and then treated with 2 U of Turbo DNase (Ambion) at 37 °C for 30 min, following by heat inactivation at 75 °C for 10 min. 10 µl of the treated RNA was used in a 20 µl qRT-PCR reaction with the qScript One-Step Fast qRT-PCR kit (Quanta Biosciences), a TaqMan probe (5′-/56-FAM/CCC ACCAACAGGCGGCCTTAACTG/36-TAMSp/-3′) (IDT) and primers designed targeting the Pol gene of JR-CSF (5′-CAATGGCAGCAATTTCACCA and 3′-GAATGCCAAATTCCTGCTTGA) or REJO.c (5′-CAATGGCCCC AATTTCATCA and 3′-GAATGCCGAATTCCTGCTTGA). Samples were run in triplicate on an Eppendorf Realplex^4 Mastercycler (Eppendorf). The following cycling conditions were used: 1 cycle of 49 °C for 5 min, 1 cycle of 95 °C for 30 s and 55 cycles of 95 °C for 3 s and 60 °C for 1 min. Virus titer was determined by comparison with a standard curve generated using RNA extracted from a serially diluted mixture of commercially titered viral stock and pure mouse serum. The limits of detection were 1,500 copies ml^{-1} and 1,000 copies ml^{-1} for JR-CSF and REJO.c, respectively. Commercial viral load assays were performed by the ARI-UCSF Laboratory of Clinical Virology using the Abbott Real-Time PCR assay with a sensitivity of 200 copies ml^{-1}.

Histological staining for HIV p24. Spleens were removed from mice, kept in 10% neutral buffered formalin for 24 h and transferred to 70% ethanol until standard paraffin embedding and processing. 4-µm-thick sections were taken, and immunohistochemical staining was performed for HIV p24 detection using the Kal-1 mouse monoclonal antibody (Vector Laboratories) at a 1:25 dilution and standard antigen retrieval techniques. The slides were examined using the Olympus BX51 light microscope, and images were obtained using a SPOT Insight Digital Camera (Diagnostic Instruments).

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