INTRODUCTION

Women are at much higher risk of acquiring HIV-1 infection through heterosexual transmission than men, and constitute more than half of all HIV/AIDS cases worldwide. While the mucosal lining of the female genital tract usually presents a robust barrier against pathogens via a variety of physical and immunologic defenses, the specific roles that vaginal, ectocervical and endocervical mucosa have in protection against HIV-1 transmission remains unknown. The stratified squamous epithelium of the vaginal and ectocervical mucosa is alleged to provide better mechanical protection against pathogens compared with the single layer columnar epithelium of the endocervix, although recent observations have suggested otherwise. HIV-transmission can also occur across the vaginal/ectocervical epithelia as well as the cervical transformation zone. Langerhans cells reside close to the surface of vaginal and ectocervical mucosae and may transfer HIV-1 to CD4+ T cells that normally reside below the epithelial layer. Epithelial cells also secrete several biologic factors that can inhibit HIV infection and migration. Damage or disruption of the epithelial layer increases the ability of HIV-1 to transfer to CD4+ T cells that normally reside below the mucosal layer and allows HIV-1 virions to access cell types permissive for infection. This process is thought to be essential for HIV-1 to establish infection as the genital epithelial cells themselves lack CD4 receptors. In addition to an effective vaccine to protect against HIV infection, development of a potent anti-HIV microbicide remains an important strategy to prevent HIV transmission. However, to date, six candidate microbicides have been found to be ineffective in phase Ib or II clinical trials, yet another clinical trial of one-daily dosing regimen with tenofovir gel failed to demonstrate any detectable efficacy in at risk women. These studies underline the need to develop additional microbicide strategies with complementary or synergistic activity. Equally important is the recognition that patient adherence to microbicide dosing regimens is critical to reducing the risk of HIV acquisition, particularly when an effective microbicide is available. The recent identification of novel highly potent human anti-HIV broadly neutralizing antibodies (BnAbs) and their further improvement by structure based design has led to intense interest in their possible use in pre-exposure prophylaxis. In addition, in the absence of an effective vaccine, vector-mediated gene transfer has received renewed interest as an immunoprophylaxis strategy to engineer secretion of existing BnAbs into the circulation. Adeno-associated virus (AAV) vectors are particularly attractive for gene delivery because of their safety and efficacy profile and the ability of different serotypes to transduce a variety of tissue and cell types. Furthermore,
transduction is potentially long lasting, directing gene expression over a period of months to years in nondoning tissues.\textsuperscript{31,33,34} For example, persistent neutralizing activity was seen for at least 24 weeks following a single intramuscular injection of AAV encoding the prototypic human anti-HIV-1 gp120 CD4 binding site BnAb, b12, into mouse muscle.\textsuperscript{35} More recently, similar delivery of further engineered self-complementary AAV vector encoding b12 immunoglobulin G (IgG) in humanized mice provided protection from infection when challenged intravenously or intravaginally with HIV-1.\textsuperscript{29,36} In addition, we have previously demonstrated that AAV vectors encoding b12 IgG1 minibodies (bivalent single-chain antibody (scFv) with Fc (scFvFc)) can transduce human primary genital epithelial cells (PGECs) and their stem cells in vitro and block HIV-1 transmigration and infection.\textsuperscript{37} This Ab format was chosen for ease of cloning, high levels of expression, maintenance of Fc effector functions and potential greater tissue penetration than IgGs owing to their smaller size. Transduction of epithelial stem cells is expected to be a key feature of persistence, as the apical layers of the cervical–vaginal mucosa continuously sheds, whereas the basal layers of the mucosa including the epithelial stem cells is maintained as a replenishing source of squamous epithelial cells. Whether persistent minibody expression from the transduced stem cell population will be for years is not known; however, even if gradual diminishing expression from the extrachromosomal AAV vector occurs over time, therapeutically meaningful local concentrations of anti-BnAbs may still be expressed for several months. Indeed, the use of AAV vectors for gene transfer to lung epithelial cells\textsuperscript{38} and their progenitors\textsuperscript{39,40} as well as other stem cell types,\textsuperscript{41-43} has recently been demonstrated. The rhesus macaque (Macaca mulatta) (Rh) model is used extensively as a surrogate for testing HIV-1 microbicides because of the many similarities between the anatomy and physiology of the human and primate genital tracts.\textsuperscript{44,45} In the present study, we evaluated multiple AAV serotypes for gene transfer to freshly immortalized endocervical, ectocervical and vaginal epithelial cell lines derived from female Rh and Rh PGECs. We performed pilot AAV-green fluorescent protein (GFP) gene transfer studies to the lower genital track of female Rh to evaluate the feasibility of stem cell gene transfer and duration of transgene expression. We also modified our procedures for AAV gene transfer to include scarification of the cervical–vaginal epithelium to better expose the basal epithelial stem cell layer. For these studies, we chose AAV-encoding b12-scFvFc minibodies as b12 IgG effectively neutralizes the chimeric R5 tropic virus SHIV162p4 \textit{in vitro}\textsuperscript{46} and protects macaques against vaginal challenge with SHIV162p4 following Ab application to the cervical–vaginal mucosa.\textsuperscript{46} Our results show that intravaginal application of AAV-6-b12 vector to female Rh resulted in sustainable detection of b12 minibodies in vaginal secretions for at least several months. AAV-anti-HIV BnAb gene transfer to the vaginal and cervical epithelium stem cell compartment represents a novel microbicide strategy that may, with further optimization, have the potential for preventing HIV-1 infection in women during heterosexual transmission.

RESULTS

Generation of Rh PGEC lines

Papillomavirus-immortalized cell lines from normal human vaginal, ectocervical and endocervical cells have been previously generated and cultured for \textit{in vitro} studies.\textsuperscript{47,48} These cell lines maintain expression of tissue-specific differentiation proteins and were similar to primary organotypic cultures.\textsuperscript{48} To evaluate transduction of corresponding Rh macaque tissues by AAV vectors, we generated immortalized Rh/V/E6E7, Rh/Ect/E6E7 and Rh/End/E6E7 cell lines from healthy Rh macaque vaginal, ectocervical and endocervical epithelia, respectively, using retroviral vector LXSN-16E6E7 transduction.\textsuperscript{48} A representative example of the immortalized vaginal cell line morphology in culture is shown in Figure 1A, in which small keratinocyte-like cells are observed by light microscopy (panel b), in contrast to the primary cell cultures (a).

![Figure 1](image-url)

**Figure 1.** (A) Light microscopic examination of the Rh macaque vaginal epithelial cell line Rh/V/E6E7 reveals small keratinocyte-like cells (b), in contrast to the primary cell cultures (a). In Ca\textsuperscript{2+}-supplemented keratinocyte serum-free medium, the immortalized cells formed tight colonies of attached cells (c and d). Doubling time of the cultures was approximately 72 h (c and d). Note that both Rh macaque (e) and human vaginal epithelial cell line (f) at the light microscopic level have a similar morphology. (B) Immunofluorescence staining of methanol-fixed Rh macaque vaginal epithelial cell line Rh/V/E6E7 with the specific epithelial cell markers anti-ck-19-PE (b) and anti-ck-10-FITC (e) antibodies. (a and d) 4′,6-Diamidino-2-phenylindole (DAPI) staining, (c) showing (a) and (b) combined and (f) showing (d) and (e) combined.
Table 1. Rh macaque genital epithelial cell lines stained with various cytokeratin-specific antibodies

| Protein          | Vaginal epithelial | Ectocervical | Endocervical |
|------------------|--------------------|--------------|--------------|
| Ck19             | ++                 | ++           | +            |
| Ck18             | –                  | +            | +            |
| Ck10             | +                  | +            | –            |
| SC (poly IgA receptor) | (+)                | –            | +            |

Abbreviations: Ck, cytokeratin; IgA, immunoglobulin A; –, no expression; +, positive expression; (+), weak expression.

Figure 2. (a and b) Transduction of Rh macaque endocervical, ectocervical and vaginal epithelial cells by various AAV serotypes expressing GFP at MOI of 2 × 10⁵ vg per cell. (a) Expression of GFP protein by transduced cells was detected by FACS, and presented as percentages of GFP+ cells. Note that AAV-2 and -6 yield the highest transduction rates. (b) Fluorescence microscopy examination of Rh macaque vaginal cell line Rh/V/E6E7 transduced with various AAV serotypes that express GFP.
After preincubating b12 minibodies or full-length b12 IgG in infectivity by b12 minibodies across a monolayer of Rh PGECs and 6 h from tissue samples treated with SHIV162p4 and b12 IgG1 antibodies, or with b12 minibodies, had almost completely lost the ability to infect TZM-bl cells.

In vitro transduction of Rh macaque genital epithelial monolayer

Monolayers of female Rh PGECs (vaginal) were transduced by AAV-6 vectors encoding the anti-HIV-1 b12 minibody or an irrelevant control minibody (5 × 10^10 particles in 100 μl) to the apical surface followed by SHIV162p4 virus (5 ng in 100 μl) at 4 days after transduction. As measured by p27 ELISA, the number of SHIV162p4 viral particles that crossed monolayers in cells transduced with AAV-6 expressing control minibody was not statistically significant at 3 and 6 h from untransduced cells (P < 0.17 and 0.39, respectively), the O.N. supernatant was higher for the untransduced cells (P = 0.002); however, at this time point, most of the virus is noninfectious.49 (Figure 4). For monolayers that were transduced with AAV-6-b12 vectors, supernatants collected from the lower chambers contained significantly less SHIV162p4 viral particles compared with the no treatment controls (P < 0.001 for each time point). In addition, supernatants from AAV-6-b12-transduced cells compared with control minibody-transduced cells also showed 79%, 81% and 83% inhibition of migration at 3 h, 6 h and overnight, compared with 16%, 26% and 48%, respectively, these changes were statistically significant (P < 0.005 for each time point). These data suggest that in vitro transduction of female Rh PGECs with AAV-6-b12 interferes with SHIV162p4 transfer through these cell monolayers.

In vivo evaluation of AAV-6-GFP transduction of Rh macaque female genital epithelial tissue

To examine whether AAV-6 could transduce genital epithelial tissue in vivo, AAV-6-GFP 0.5 × 10^12 genomic copies (gc) per animal (the approximate estimation of the MOI is 1 × 10^7 per cell) in phosphate-buffered saline (PBS) were instilled into the vaginal vault of two female AAV-6-seronegative Rh. We chose AAV-6-seronegative animals to avoid any interaction between anti-AAV-6 antibodies and the AAV-6-GFP vectors, as at this early stage of investigation we did not know what effect prior anti-AAV-6 immunity may have on the transduction efficiency and overall results. No attempt was made to remove the mucosal secretions or expose the basal epithelial layer by scarification. Vaginal and ectocervical biopsies were collected before transduction and at 1, 4 and 8 weeks after transduction to evaluate GFP expression. GFP expression was observed in the cytoplasm of all layers of the cervical epithelium on day 7 in one animal, including an occasional p63+ stem cell in the basal layer (Figure 5). No GFP+ cells were observed in the biopsy sections from 4 or 8 weeks after transduction; however, only two 1 mm × 1 mm blind biopsies from the vagina and two from the ectocervix were available for examination. These results indicated that AAV-6-GFP is able to transduce genital epithelial tissue in female Rh in vivo.

Local secretion of b12 minibodies after vaginal application of AAV-6-b12 vectors in vivo

Next, the capacity of AAV-6-b12 vectors to induce local secretion of b12 minibodies following Rh vaginal application of AAV-6-b12 vectors (0.5 × 10^12 gc per animal) was investigated in two animals. Immediately before application, the superficial epithelial cervical and vaginal mucosal layers were disrupted with a standard Papanicolaou’s cervical smear brush to enhance penetration of the vector to deeper cell layers and potentially prolong transgene expression. Vaginal fluids obtained from Weck-Cel wicks at varying times after transduction were recovered and b12 minibodies were detectable at concentrations ranging between 450 and 800 pg ml^−1 (Figure 6). These results demonstrate that a single application of AAV-6-b12 vector has the capacity to transduce the lower genital tract of female Rh and to induce the secretion of b12 minibodies. Moreover, b12 minibodies were detectable over the 79-day experiment. These in vivo findings suggest that AAV-based gene transfer of BnAb b12 to the lower genital tract of female Rh could provide prolonged protection against an intravaginal SHIV162p4 challenge.
DISCUSSION

Epithelial cells of the cervical–vaginal mucosa provide the initial physical defense against HIV-1 infection. However, the protection offered by these cells is sometimes incomplete. Thus, enhancing anti-HIV-1 immunity at the mucosal cell surface by local secretion of anti-HIV-1 BnAbs to block HIV-1 entry would provide an important new intervention that could slow the spread of HIV/AIDS. To that end, we constructed immortalized Rh vaginal, ectocervical and endocervical cell lines and determined that AAV serotypes 2 and 6 allowed the highest level of transduction efficiency. We also conducted proof-of-principle transduction studies on female Rh and demonstrated sustained detection of b12 minibodies in vaginal secretions following preconditioning with depoprovera and topical application of the AAV-6-b12 vector. Depoprovera treatment was performed to reduce the thickness of the mucosal epithelial layers with the expectation that this would both facilitate AAV-6 gene transfer and allow in future studies efficient SHIV162p4 transmission in control treated animals. In two Rh, AAV-6-GFP transduction and transgene expression was initially studied. Biopsy analysis of the ectocervical and vaginal mucosa demonstrated GFP expression at 7 days after topical application of the pseudovirus to the vaginal mucosa with the occasional transduction of p63+ stem cells also being seen (Figure 5e). GFP expression was not identified at time points after 7 days in the two monkeys, although only a small proportion of the ectocervix and vagina was evaluated. Indeed, the small blind biopsies limited our ability to assess quantitatively stem cell transduction frequency. This limitation may be overcome by better visibility of the cervical–vaginal mucosa using bioluminescence reporter viruses and/or multicolor fluorescence minendoscopic imaging. Indeed, the latter technique was used to follow GFP/RFP expression in mice over 7 days following intravaginal transduction with papilloma GFP pseudoviruses, although the stem cell compartment was not examined. Additionally, no attempt was made in these two Rh to prepare the epithelial surface for gene transfer by removal of the protective mucosal secretions.

In two subsequently treated Rh and in order for the AAV-6-b12 vector to gain potentially easier access to the basal epithelial layers that contains p63+ stem cells, the mucosal surface was lightly scarified before AAV-6-b12 transduction using a standard Pap smear brush. A single application of $0.5 \times 10^{12}$ gc per animal AAV-6-b12 resulted in a peak of $600–800$ ng ml$^{-1}$ b12 minibody expression in the vaginal secretions at 7 days after transduction. In addition, the b12 minibody was detected 60–70% of peak levels throughout the 79-day study. This result suggests that successful AAV-6 transduction had occurred at the mucosal surface, presumably including exposed epithelial stem cells (Figure 5e); however, additional studies will be required to quantify the transduction efficiency of different cell populations within the lower reproductive tract. Further refinements to the AAV vector designs and/or enhancements in delivery with formulations that...
are known to improve expression and transduction efficiency,\textsuperscript{53,54} as well as the possible use of tyrosine-modified RAAV vectors,\textsuperscript{55–57} and directed evolution to increase stem cell gene transfer efficiencies.\textsuperscript{57,58} Will likely improve these results. Local topical delivery of the therapy with only mild scarification equivalent to a pap smear as the preparation provides a means by which reapplication, if and when required as determined by quantitative analysis (e.g. titers by wicks), could provide better compliance than daily pericoital microbicide applications.

We anticipate application of higher doses such as 10\textsuperscript{13} or 10\textsuperscript{14} gc per animal may yield a much higher concentration of b12 minibinaries in vaginal secretions. However, the current concentration may be capable of blocking infection if translated into protection against HIV infection in humans, as most human infection via sexual encounter probably involves repeated exposures to much lower doses of virus than we used in the in vitro assays (5 ng p27 or 95 tissue culture infectious dose 50). In addition, it has been reported that lower amounts of antibody than previously considered protective may provide benefit in the context of typical human exposure to HIV-1.\textsuperscript{59} The antibody secretion titers that we observed in the present study are approaching or may have reached therapeutically relevant concentrations for the more potent human antibodies against the CD4 binding site or other potent neutralization epitopes.\textsuperscript{27–29}

While previous microbicide studies with female Rh,\textsuperscript{46,50} have demonstrated that intravaginal instillation of high concentrations of b12 IgG can afford protection against SHIV challenge, it is unknown whether the prolonged secretion of lower levels of b12 minibodies will saturate the local tissues and provide levels that are adequate to provide sustained protection against intravaginal SHIV162p4 virus challenge. In addition, in the present work, we used b12 IgG1 minibodies to establish proof of principle that genes encoding BnAb can be delivered to the lower genital tract of female Rh via AAV-based vectors. However, systemic protein delivery and bone marrow stem cell gene transfer approaches in humanized mice to deliver a dimeric form of b12 IgA2 showed superiority over b12 IgG1 in providing protection against intravaginal HIV-1 challenge.\textsuperscript{50} Therefore, future studies should evaluate combinations of the newly reported potent BnAbs antibodies,\textsuperscript{28,61} as well as the use of IgA isotype to achieve a wider protection against HIV-1 isolates.\textsuperscript{50}

Despite the positive results of using AAV vectors in a number of preclinical and clinical settings, the pre-existing and/or recall immune responses to the wild-type virus from which the vector is engineered may raise some concerns about safety as well as the therapeutic efficacy. AAV-2 is the most seroprevalent in the human population, whereas seropositivity to AAV-6 is reported to be lower but it is also less studied.\textsuperscript{62–65} In our approach, we used AAV-6 vectors that were found to be resistant to the neutralizing effects of anti-AAV-2 antibodies.\textsuperscript{66} Whether induction of local anti-AAV immunity will negatively impact repeated topical delivery and BnAb expression will require further evaluation. However, anti-AAV antibodies failed to block muscle transduction when the vector was directly injected intramuscularly and the development of AAV antibody titers with not consultation AAV antibodies to the transgene.\textsuperscript{34,67,68} Readministration of AAV-2/9 in the presence of high levels of circulating neutralizing antibodies also had minimal effect on transgene expression.\textsuperscript{40} In addition, several strategies are under investigation to mitigate immune-mediated interference of AAV transgene delivery such as blockade of the TLR9-MyD88-type I IFN pathway or using empty vectors as decoys.\textsuperscript{59–71} Another safety concern is based on detection of AAV DNA in human genital tissues and in material from spontaneous abortions.\textsuperscript{72,73} In a more recent study, the presence of AAV DNA in genital specimens was not found to be associated with clinically relevant infertility; however, longitudinal studies may be required to clarify previous suggestions of an influence of AAV infection on early pregnancy problems.\textsuperscript{74–76}

The lack of an effective prophylactic HIV-1 vaccine has led to increased interest in anti-HIV-1 agents that can be applied topically to prevent mucosal transmission during sexual activity. A variety of compounds have been proposed as potential topical anti-HIV microbicides;\textsuperscript{53,57–58} however, to date, no agent has been shown to be effective in conferring protection against HIV-1 infection with most agents failing during clinical trials. A notable exception is tenofovir gel (CAPRISA-004), which showed marginal but statistically significant protection against HIV-1 in a clinical trial.\textsuperscript{5} This trial also highlighted compliance issues with agents that required daily or timed application, and identified decreased adherence to product instructions over time by study participants. Such behavior-related issues may be pre-empted by the use of anti-HIV agents that have more sustained activity. While the benefit of using AAV-mediated anti-HIV-neutralizing antibody gene transfer by systemic intravenous delivery has recently been demonstrated to provide durable protection against HIV-1 infection,\textsuperscript{29,36} the potential safety issues of systemically transducing a wide variety of host tissues remains unknown. Our findings provide a proof of principle that AAV vector transduction of cervical-vaginal epithelial cells and their stem cells can lead to local and long-term secretion of a potent and broadly neutralizing anti-HIV gp120 minibody over at least several months, thus bypassing the need for daily use. The potential effects, if any, of local anti-AAV immunity on minibody expression will still need to be fully evaluated. Accordingly, our data provide justification for moving this approach toward an in vivo protection study in the macaque model to determine if AAV-6-BnAb gene transfer to the lower genital tract of female Rh can lead to the secretion of protective levels of neutralizing b12 Ab and prevent infection following intravaginal SHIV162p4 challenge. Our study thus represents a novel HIV-1 microbicide strategy and potential preventative agent for HIV-1 transmission to women.

\section*{MATERIALS AND METHODS}

\subsection*{Growth media}

Rh macaque female genital epithelial cells were cultured in keratinocyte serum-free medium (Gibco/BRL Life Technologies, Grand Island, NY, USA), supplemented with bovine pituitary extract and recombinant human epidermal growth factor. The medium was further supplemented with 10\textsuperscript{5} U ml\textsuperscript{−1} penicillin, 100 \textmu g ml\textsuperscript{−1} streptomycin and CaCl\textsubscript{2}; to a final calcium concentration of 0.4 mM. All other cell lines used in this study were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA).

\subsection*{Cell lines and viruses}

The TZM-bl cell line, acquired from the National Institutes of Health AIDS Research and Reference Reagent Program (NIH-ARRRP, Germantown, MD, USA), is a CCR5+ HeLa cell line that expresses CD4 and HIV-1 coreceptor CCR5; it also contains integrated reporter genes for luciferase and Escherichia coli β-galactosidase, under the control of an HIV long-terminal repeat sequence (lTR gene), which allows for quantification of HIV infection. PA317, 293 T and COS-1 cells were purchased from ATCC (Manassas, VA, USA). All cells and cultures were maintained at 37 °C in a 5% CO\textsubscript{2} humidified incubator. The R-tropic SHIV162p4 virus is based on molecular clones of SIVmac239 and the RS HIV-1 primary isolate SF162 (derived from in vivo after three serial passages in Rh macaques,\textsuperscript{79} which was a gift from Dr Cheng-Meyer C (Aaron Diamond AIDS Research Center, New York, NY, USA)).

Establishing Rh macaque PGEC lines

Fresh Rh endocervical, ectocervical and vaginal tissues were obtained as biopsies from the New England Primate Research Center, Harvard Medical School or as explants from Tulane Primate Research Center in accordance with IACUC regulations. Tissues were collected in cold Hanks’ balanced salt solution without Ca\textsuperscript{2+} and Mg\textsuperscript{2+} and supplemented with penicillin (100 U ml\textsuperscript{−1}), streptomycin (100 \textmu g ml\textsuperscript{−1}) and gentamicin.
(50 μg ml⁻¹) (Gibco), and epithelial cells were isolated using a modified previously described protocol. Briefly, tissue was minced into very small pieces and then digested for 3 h at 37 °C in 1 mg ml⁻¹ of collagenase dispase containing 1 mg ml⁻¹ of DNase (Sigma, St Louis, MO, USA), with gentle stirring; the mixture was then passed through a cell strainer (250 μm), spun down (1500 r.p.m.) for 20 min and resuspended in Dulbecco's modified Eagle's medium with 10% fetal calf serum. After additional centrifugation, the pellet was resuspended in epithelial cell selection medium (keratinocyte serum-free medium) in T-25 flasks; the cultures were fed every 3 days for the next 6–9 days, and subcultured to expand for cryopreservation and to set up cultures. Cells were passaged two times before transduction with LXSN-16E6E7 retroviral vector packaged by the amphotropic fibroblasts line (PA317, Briefly, 1 ml of (LXSN-16E6E7) supernatant was used to transduce the Rhesus primary cells and then cultured in G418 selection media. Immortalized cells were then cultured in Ca²⁺-supplemented (0.4 mM CaCl₂) keratinocyte serum-free medium, and cell stocks of the generated ‘primary’ cell lines from vaginal, ectocervical and endocervical tissues were cryopreserved in liquid nitrogen or at –80 °C after the second passage by freezing in 90% calf serum (HyClone, Logan, UT, USA) and 10% dimethyl sulfoxide (Sigma).

Animal inoculations
All animal procedures including euthanasia were performed in accordance with guidelines and recommendations of The Guide for the Care and Use of Animals, the standards of the Harvard Medical School Standing Committee on Animals and The Association for the Assessment and Accreditation of Laboratory Animal Care. Adult female Rh were confirmed serologically negative for AAV-1 and –6 before inoculation, and were treated with one-monthly subcutaneous Depo-Provera. Throughout the study period beginning 2 months before initial treatment. Animals were sedated using standard procedures, placed in ventral recumbency with hips elevated, followed without (AAV-6-GFP) or with (AAV-6-b12) pretreatment by gentle abrasion of the vaginal and cervical mucosa with a Pap smear cervical brush (Kansas Pathology Consultants, Wichita, KS, USA). No spectrum was used to avoid loss of transduction fluid. For preparation of AAV-6-b12 transduction, the cervical brush was inserted blindly and used to scarify the vagina/ectocervix. Next, Rh were intravaginally inoculated with a total volume of 0.5 ml per animal of AAV-6-GFP or AAV-6-b12 (0.5 x 10⁻³ gc diluted in PBS) using a 1 ml syringe that was inserted as deep as possible into the vagina. Hip elevation was maintained for 30 min to allow for complete absorption of the vector; no leakage was observed during or following the inoculation period. Blood samples and vaginal secretions were collected up to once a week throughout the study period, and spectrum-guided cervicovaginal biopsies were obtained at weeks –1 (preinoculation), 1, 4 and 8.

Production of AAV serotypes expressing GFP and b12 minibody
AAV serotypes 1, 2, 5, 6, 8 and 9 expressing GFP were produced at Harvard Gene Therapy Initiative (Harvard Institute of Medicine, Boston, MA, USA) and Penn Vector Core (University of Pennsylvania, Philadelphia, PA, USA), whereas AAV serotypes 3 and 4 expressing GFP were obtained from the AAV core at the University of North Carolina (Chapel Hill, NC, USA). AAV-6 expressing b12 minibody was obtained commercially (Virapur LLC, San Diego, CA, USA).

Transduction of cells, flow cytometry analysis and fluorescence microscopy
For AAV transduction, 5 x 10⁵ Rh/V/E6E7, Rh/Ect/E6E7 and Rh/End/E6E7 immortalized cells were incubated in 24-well plates for 4 h with AAV (1, 2, 3, 4, 5, 6, 8 or 9) expressing GFP (10⁻⁷ gc and at MOI of 2 x 10⁻⁶ vg per cell). The medium was replaced, and the cells were examined on day 3. Expression of GFP protein was detected by flow cytometry (FACS Calibur; Becton Dickinson, Rutherford, NJ, USA) and was represented as the percentage of GFP⁺ cells, and was also assessed visually by fluorescence microscopy.

Preparation of monolayers of Rh macaque cells
Rh PGECs were seeded at a density of 10⁵ cells per well in the upper chambers of 12 mm diameter transwells with 3 μm pore size polycarbonate membranes, and cultured at 37 °C. Cells were then fed every 2 days until tight junctions formed between the cells. This was determined by measuring the changes in mechanical tension in the cell monolayer using transendothelial electrical resistance (volt/ohm meter equipped with an electrode Millicell ERS; Millipore) (typically the tight junctions formed between days 6 and 8 after plating are about 600 ohm m⁻²). The cell monolayer on the filter effectively divided the well into an apical compartment and a basal compartment. To ensure the integrity of the Rh PGECs barrier, we monitored the elevated transendothelial electrical resistance of each cell monolayer, which must exceed > 600 ohm m⁻² and also measured the paracellular passage of Dextran-Rhodamine B (70 kDa).

Preparation of AAV-6-b12 minibodies
AAV stocks of the generated AAV-6-b12 minibodies was measured using a human IgG ELISA kit (Bethyl Laboratories, Montgomery, TX, USA). Ninety-six-well microtiter plates were coated overnight at 4 °C with 10 ng per well of HIV-1 bal gp120 (NIH-ARRRP, Germantown, MD, USA; cat. no. 4961) in 0.05 M carbonate–bicarbonate buffer (pH 9.6, Sigma) and blocked in PBS (1% bovine serum albumin) for 1 h; serial dilutions of b12 minibodies were added to the plate for 1 h at room temperature. After washing, horseradish peroxidase-conjugated, affinity-purified goat anti-human IgG (Bethyl Laboratories, Montgomery, TX, USA) was added (1:50 000) for 1 h. After extensive washing, the plate was developed by the addition of TMB substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) and detected by reading the absorbance (OD) at 450 nm.

Preparation of monolayers of Rh macaque cells
Rh PGECs were seeded at a density of 10⁵ cells per well in the upper chambers of 12 mm diameter transwells with 3 μm pore size polycarbonate membranes, and cultured at 37 °C. Cells were then fed every 2 days until tight junctions formed between the cells. This was determined by measuring the changes in mechanical tension in the cell monolayer using transendothelial electrical resistance (volt/ohm meter equipped with an electrode Millicell ERS; Millipore) (typically the tight junctions formed between days 6 and 8 after plating are about 600 ohm m⁻²). The cell monolayer on the filter effectively divided the well into an apical compartment and a basal compartment. To ensure the integrity of the Rh PGECs barrier, we monitored the elevated transendothelial electrical resistance of each cell monolayer, which must exceed > 600 ohm m⁻² and also measured the paracellular passage of Dextran-Rhodamine B (70 kDa).

SIV p27 antigen-capture ELISA assay
SIV/HIV-162p4 viral particles that crossed the Rh macaque monolayer to the lower chambers of the transwell cultures were measured by SIV p27 antigen-capture ELISA assay (Advanced Bioscience Laboratories Inc., Kensington, MD, USA).

Measurement of viral infectivity
TZM-bl cells that contain a luciferase gene under the control of the HIV-1 LTR promoter were seeded in 96-well plates (4000 cells per well) and grown overnight. The medium was then removed, and cells incubated with 100 μl of media were collected at different time points (up to 24 h) and added to 12 mm diameter transwells. Supernatant was measured using the dual-luciferase system (Promega, Madison, WI, USA; cat. no. E1501) and measured using the Centro LB 960 Luminometer (Berthold, Bad Wildbad, Germany).

MSD assay
This assay in principle is similar to an ELISA assay with the outcome measured with Meso Scale Discovery (MSD, Rockville, MD, USA) technology, which is based on electrochemiluminescence detection. We used a Sulfo-Tag label that emits light upon electrochemical stimulation. Briefly, each well of a 96-well plate was coated with 5 μl of HIV-1 gp120 bal protein at 40 μg ml⁻¹, and incubated overnight at 4 °C. The following day, the antigen-coated plate was incubated at 37 °C for 1 h with 2% bovine serum albumin blocking agent. Plates were washed with 0.05% PBS-T, and 25 μl of the diluted vaginal secretion samples were added to each well. After 1 h incubation at 37 °C, plates were washed with 0.05% PBS-T, and 25 μl (500 μg ml⁻¹) of Sulfo-Tag labeled goat anti-human IgG secondary antibody (Meso Scale Discovery, Gaithersburg, MD, USA; cat. no. R32A2J-1) was added to each well. The plates were incubated again for 1 h at 37 °C, washed and then 150 μl of MSD Read Buffer-T 4X (with surfactant) (diluted 1:4 in water) was added to each well. The plates were read using an MSD sector imager, Model no. 2400.
Immunofluorescence and immunochrometry

Immunofluorescence analysis was performed on Rh ectxcervical, endocervical and vaginal immortalized cell lines by growing them for 3 days on 13 mm Thermofax coverslips in 24-well Falcon tissue culture plates with keratinocyte serum-free medium (Gibco) (Nunc, Naperville, IL, USA; Becton Dickinson). Cells on coverslips were fixed with cold absolute methanol for 5 min and quickly rinsed with distilled water. The cell lines were then phenotyped using the specific epithelial cell markers, CK19, CK18 and CK10, and secretory component (polo lgA receptor) monoclonal antibodies. Cells were then examined under fluorescence microscope. Immunochrometry: Vaginal and cervical biopsies from normal Rh or Rh intravaginally transduced with AAV-6-GFP were fixed in 2% paraformaldehyde for 2 h before being cryopreserved in 30% sucrose, embedded in Tissue-Tek cryo OCT compound (Thermo Scientific), frozen in 2-methylbutan (Fisher, Pittsburgh, PA, USA) and stored at − 80 °C. Blocks were cryosectioned at 5 μm and processed to visualize the basal epithelial layer (marker p63) with the GFP. Sections were incubated with the primary antibody for p63 (1:200, cat. no. sc-8431; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 30 min followed by biotinylated goat anti-mouse IgG (1:200, cat. no. BA9200; Vector Laboratories Inc., Burlingame, CA, USA) and streptavidin 488 (1:500, cat. no. S-11223; Life Technology) for 30 min each. Nuclei were stained with 4,6-diamidino-2-phenylindole in the mounting media. Controls included isotype-matched irrelevant antibodies. Tissues were then examined by fluorescence microscopy for GFP expression, using a Leica SPS Inverted Laser Scanning Confocal Microscope (Leica Microsystems, Buffalo Grove, IL, USA) with further image processing using the Imagej software (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

All statistical evaluations were performed using two-sample t-test. P < 0.01 was considered statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Dennis Burton (The Scripps Research Institute, La Jolla, CA, USA) for providing the scFv b12DNA plasmid, and Leonidas Stamatatos for providing the SHIV162p4 virus. We thank clinical staff Joshua Kramer, Amber Hoggatt and Matt Prockop for veterinary services and Karen Boisvert for microscopy and immunofluorescence assistance. This work was supported by NIH R21/R33 AI079767 (to WAM), by the New England Primate Research Center Base Grant P510D011103-51 (NEPRC) and the T32 Training Grant T32OD011064 (to SW).

REFERENCES

1. Parker RD, Ruutel K. A surveillance report of HIV status and high risk behaviors among rapid testing participants in Tallinn, Estonia. AIDS Behav 2011; 15: 761–766.
2. Hladik F, Hope TJ. HIV infection of the genital mucosa in women. Curr HIV/AIDS Rep 2009; 6: 20–28.
3. Carias A, McCoombes S, McRaven M, Anderson M, Galloway N, Vandergrift N et al. Initial events in establishing vaginal entry and infection by human immunodeficiency virus type-1. Immunity 2007; 26: 257–270.
4. Turville SG, Peretti S, Pope M. Lymphocyte-dendritic cell interactions and mucosal acquisition of HIV infection. Curr Opin HIV AIDS 2006; 1: 3–9.
5. Abdool Karim Q, Abdool Karim SS, Frohlich JA, Grobler AC, Baxter C, Mansoor LE et al. Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. Science 2010; 329: 1168–1174.
6. Feldblum PJ, Adeiga A, Bakare R, Wewil S, Lendvay A, Obadaki F et al. SAVVY vaginal gel (C31G) for prevention of HIV infection: a randomized controlled trial in Nigeria. PLoS One 2008; 3: e1474.
7. Halperm V, Ogunsola F, Obunge O, Wang CH, Onyejepu N, Oduboyo O et al. Effectiveness of cellulose sulfate vaginal gel for the prevention of HIV infection: results of a phase III trial in Nigeria. PLoS One 2008; 3: e3784.
8. McCormack S, Ramjee G, Kamali A, Rees H, Crook AM, Gafos M et al. PRO2000 vaginal gel for prevention of HIV-1 infection (Microbicides Development Programme 31): a phase 3, randomised, double-blind, parallel-group trial. Lancet 2010; 376: 1329–1337.
9. Peterson L, Nanda K, Opoku BK, Ampofo WK, Owusu-Amoako M, Boakye AY et al. SAVVY (C31G) gel for prevention of HIV infection in women: a Phase 3, double-blind, randomized, placebo-controlled trial in Ghana. PLoS One 2007; 2: e1312.
10. Skoler-Karpoff S, Ramjee G, Ahmed K, Altini L, Plagnias MG, Friedland B et al. Efficacy of Carraguard for prevention of HIV infection in women in South Africa: a randomised, double-blind, placebo-controlled trial. Lancet 2008; 372: 1977–1987.
11. Van Damme L, Govinden R, Mirembe FM, Guedou F, Solomon S, Becker ML et al. Lack of effectiveness of cellulose sulfate gel for the prevention of vaginal HIV transmission. N Engl J Med 2008; 359: 463–472.
12. Van Damme L, Ramjee G, Alary M, Vuyel rakebe E, Chandyeye V, Rees H et al. Effectiveness of COL-1492, a nonoxynol-9 vaginal gel, on HIV-1 transmission in female sex workers: a randomised controlled trial. Lancet 2002; 360: 971–977.
13. Heise LL, Watts C, Foss A, Trussell J, Vickerman P, Hayes R et al. Apples and oranges? Interpreting success in HIV prevention trials. Contraception 2011; 83: 10–15.
14. Hankins CA, Dybul MR. The promise of pre-exposure prophylaxis with anti-retroviral drugs to prevent HIV transmission: a review. Curr Opin HIV AIDS 2013; 8: 50–58.
15. Dey B, Lagenaar LA, Lusso P. Protein-based HIV-1 microbicides. Curr HIV Res 2011; 9: 576–594.
16. Masse BR, Boly MC, Dimitrov D, Desai K. Efficacy dilution in randomized placebo-controlled vaginal microbicide trials. Emerg Themes Epidemiol 2009; 6: 5.
17. Pace CS, Song R, Ochsenbauer C, Andrews CD, Franco D, Yu J et al. Bispecific antibodies directed to CD4 domain 2 and HIV envelope exhibit exceptional breadth and picomolar potency against HIV-1. Proc Natl Acad Sci USA 2013; 110: 15340–15345.
18. Walker LM, Huber M, Doreos KJ, Falkowska E, Pejchal R, Julien JP et al. Broad neutralization coverage of HIV by multiple highly potent antibodies. Nature 2011; 477: 466–470.
19. Balazs AB, Chen J, Hong CM, Rao DS, Yang L, Baltimore D. Antibody-based protection against HIV infection by vectored immunoprophylaxis. Nature 2012; 481: 81–84.
20. Clark KR. Recent advances in recombinant adeno-associated virus vector production. Kidney Int 2002; 61(Suppl):59–15.
21. Johnson PR, Schnepf BC, Zhang J, Connell MJ, Greene SM, Yuste E et al. Vector-mediated gene transfer engenders long-lived neutralizing activity and protection against SIV infection in monkeys. Nat Med 2009; 15: 901–906.
22. Schultz BR, Chamberlain JS. Recombinant adeno-associated virus transcription and integration. Mol Ther 2008; 16: 1175–1199.
23. Xiao PJ, Lentz TB, Samulski RJ. Recombinant adeno-associated virus: clinical application and development as a gene-therapy vector. Therap Deliv 2012; 3: 835–856.
24. Brantly ML, Chulay JD, Wang L, Mueller C, Humphries M, Spencer LT et al. Sustained transgene expression despite T lymphocyte responses in a clinical trial of rAAV-AAT gene therapy. Proc Natl Acad Sci USA 2009; 106: 16363–16368.
et al.

41 Asuri P, Bartel MA, Vazin T, Jang JH, Wong TB, Schaffer DV. Directed evolution of novel adeno-associated viruses for therapeutic gene delivery. Gene Therapy 2012; 19: 694–700.

59 Hessell AJ, Poignard P, Hunter M, Hangartner L, Tehrani DM, Bleeker WK et al. Effective, low-titer antibody protection against low-dose repeated mucosal SHIV challenge in macaques. Nat Med 2009; 15: 951–956.

60 Emeril P, Patel SN, Shimizu S, Rao DS, Gnanapragasam PN, An DS et al. Inhibitory effect of HIV-specific neutralizing IgA on mucosal transmission of HIV in humanized mice. Blood 2012; 120: 4571–4582.

61 Klein F, Halper-Stromberg A, Horwitz JA, Greul H, Scheid JF, Bournazos S et al. HIV therapy by a combination of broadly neutralizing antibodies in humanized mice. Nature 2012; 492: 118–122.

62 Bouton S, Montheillet V, Veron P, Leborgne C, Benveniste O, Montus MF et al. Prevalence of serum IgG and neutralizing factors against adeno-associated virus (AAV) types 1, 2, 5, 6, 8, and 9 in the healthy population: implications for gene therapy using AAV vectors. Hum Gene Ther 2010; 21: 704–712.

63 Calcedo R, Vandenberghhe LH, Gao G, Lin J, Wilson JM. Worldwide epidemiology of neutralizing antibodies to adeno-associated viruses. J Infect Dis 2009; 199: 381–390.

64 Louis Jeune V, Joergensen JA, Hajjar RJ, Weber T. Pre-existing anti-adeno-associated virus antibodies as a challenge in AAV gene therapy. Hum Gene Ther Methods 2013; 24: 59–67.

65 Montheillet V, Seheb S, Boutin S, Leborgne C, Veron P, Montus MF et al. A 10 patient case report on the impact of plasmapheresis upon neutralizing factors against adeno-associated virus (AAV) types 1, 2, 6, and 8. Mol Ther 2011; 19: 2084–2091.

66 Rutledge EA, Halbert CL, Russell DW. Infectious clones and vectors derived from adeno-associated virus (AAV) serotypes other than AAV type 2. J Virol 1998; 72: 309–319.

67 Manna CS, Chew A, Hutchison S, Larson PJ, Herzog RW, Arruda VR et al. AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. Blood 2003; 101: 2963–2972.

68 Stroes ES, Nierman MC, Meulenberg JJ, Franssen R, Twisk J, Henny CP et al. Intramuscular administration of AAV1-lipoprotein lipase S447X lowers triglycerides in lipoprotein lipase-deficient patients. Arterioscler Thromb Vasc Biol 2008; 28: 2303–2304.

69 Hareendran S, Baijal Krishnan S, Ben D, Kumar S, Srivastava A, Jayadhandran GR. Adeno-associated virus (AAV) vectors in gene therapy: immune challenges and strategies to circumvent them. Rev Med Virol 2013; 23: 399–413.

70 Mingozzi F, Anguela XM, Pavani G, Chen Y, Davidson RJ, Hui DJ et al. Overcoming preexisting humoral immunity to AAV using capsid decoys. Sci Transl Med 2013; 5: 194ra92.

71 Zhu J, Huang X, Yang Y. The TRLP-MyD88 pathway is critical for adaptive immune responses to adeno-associated virus gene therapy vectors in mice. J Clin Invest 2011; 120: 209–215.

72 Malhomme O, Duret JL, Abramovici-Moraes E, Schlehofer JR, Dupressoir T. Human genital tissues containing DNA of adeno-associated virus type 1-inhibitory peptides based on the antimicrobial peptide database. Antimicrob Agents Chemother 2004; 48: 5319–5323.

73 Tobiasch E, Rabreau M, Geletneky K, Larue-Charlus S, Severin F, Becker N et al. Detection of adeno-associated virus DNA in human genital tissue in and material from spontaneous abortion. J Med Virol 1994; 44: 215–222.

74 Schlehofer JR, Boeke C, Reuland M, Eggert-Kruse W. Presence of DNA of adeno-associated virus antibodies as a challenge in AAV gene therapy. Nat Med 2012; 15: 1003–1005.

75 Dereuddre-Bosquet N, Moro-Plantier A, Brouwers J, Augustijns P, Bouchemal T. Human genital tissues containing DNA of adeno-associated virus in subfertile couples, but no association with fertility and pregnancy. J Med Virol 2012; 84: 1577–1587.

76 Hessell AJ, Poignard P, Hunter M, Hangartner L, Tehrani DM, Bleeker WK et al. Effective, low-titer antibody protection against low-dose repeated mucosal SHIV challenge in macaques. Nat Med 2009; 15: 951–956.

77 Wang G, Watson KM, Peterkofsky A, Buckheit RW Jr. Identification of novel human immunodeficiency virus type 1-inhibitory peptides based on the antimicrobial peptide database. Antimicrob Agents Chemother 2010; 54: 1343–1346.

78 Yamamoto HS, Xu Q, Fichorova RN. Homeostatic properties of Lactobacillus jensenii engineered as a live vaginal anti-HIV microbicide. BMC Microbiol 2013; 13: 4.

79 Harouse JM, Gettie A, Tan RC, Blanchard J, Cheng-Mayer C. Distinct pathogenic sequelae in rhesus macaques infected with CCR5 or CXCR4 using SHIVs. Science 1999; 284: 816–819.