HHS Public Access
Author manuscript
Gene Ther. Author manuscript; available in PMC 2017 July 27.

Published in final edited form as:
Gene Ther. 2014 September ; 21(9): 802–810. doi:10.1038/gt.2014.56.

Prolonged Expression of an Anti-HIV-1 gp120 Minibody to the Female Rhesus Macaque Lower Genital Tract by AAV Gene Transfer

Ussama M. Abdel-Motal[1], **Carole Harbison**[2], **Thomas Han**[1], **Jeffrey Pudney**[3], **Deborah J. Anderson**[3], **Quan Zhu**[1], **Susan Westmoreland**[2], and **Wayne A. Marasco**[1,*]

[1]Department of Cancer Immunology & AIDS, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02215, USA

[2]Division of Comparative Pathology, New England Primate Research Center, Harvard Medical School, Southborough, MA, USA

[3]Department of Obstetrics and Gynecology, School of Medicine, Boston University, Boston, MA

Abstract

Topical microbicides are a leading strategy for prevention of HIV mucosal infection to women, however, numerous pharmacokinetic limitations associated with coitally-related dosing strategy have contributed to their limited success. Here we test the hypothesis that adeno-associated virus (AAV) mediated delivery of the b12 human anti-HIV-1 gp120 minibody gene to the lower genital tract of female rhesus macaques (Rh) can provide prolonged expression of b12 minibodies in the cervical-vaginal secretions. Gene transfer studies demonstrated that, of various GFP-expressing AAV serotypes, AAV-6 most efficiently transduced freshly immortalized and primary genital epithelial cells (PGECs) of female Rh *in vitro*. In addition, AAV-6-b12 minibody transduction of Rh PGECs led to inhibition of SHIV162p4 transmigration and virus infectivity *in vitro*. AAV-6-GFP could also successfully transduce vaginal epithelial cells of Rh when applied intra-vaginally, including p63+ epithelial stem cells. Moreover, intra-vaginal application of AAV-6-b12 to female Rh resulted in prolonged minibody detection in their vaginal secretions throughout the 79 day study period. These data provide proof-of-principle that AAV-6-mediated delivery of anti-HIV broadly neutralizing antibody (BnAb) genes to the lower genital tract of female Rh results in persistent minibody detection for several months. This strategy offers promise that an anti-HIV-1 genetic microbicide strategy may be possible in which topical application of AAV vector, with periodic reapplication as needed, may provide sustained local BnAb expression and protection.

Keywords

Gene therapy; AIDS; epithelial stem cells; AAV vectors; rhesus macaque; HIV-1; microbicide

*Corresponding author: Wayne A. Marasco, M.D., Ph.D. Department of Cancer Immunology & AIDS Dana-Farber Cancer Institute, Harvard Medical School 450 Brookline Ave. JF 824 Boston, MA 02215, USA Tel: 617-632-2153 Fax:: 617-632-3113 wayne_marasco@dfci.harvard.edu.

The authors declare no conflict of interest.

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.[1] 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 play 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 to the single layer columnar epithelium of the endocervix[2], although recent observations have suggested otherwise.[3] HIV-1 transmission can also occur across the vaginal/ectocervical epithelia as well as the cervical transformation zone.[4-6] Langerhan's 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 biological factors that can inhibit HIV infection and migration [7-12]. Damage or disruption of the epithelial layer increases the ability of HIV-1 to penetrate the mucosal layer and allows HIV-1 virions to access to cell types permissive for infection.[13, 14] 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 IIb or III clinical trials.[15-22] The CAPRISA 004 phase IIb trial provided proof of concept for vaginal microbicides, demonstrating that 1% tenofovir microbicide gel reduced the risk of HIV acquisition for women by 39% overall, and by 54% in women reporting >80% adherence to the dosing regimen[15, 23], yet another clinical trial of one-daily dosing regimen with tenofovir gel failed to demonstrate any detectable efficacy in at risk women[24]. These studies underline the need to develop additional microbicide strategies with complementary or synergistic activity [25]. 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.[26]

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.[27, 28] 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.[29-31] Adeno-associated virus (AAV) vectors are particularly attractive for therapeutic antibody (Ab) gene delivery[29, 31] because of their safety and efficacy profile[32, 33] and the ability of different serotypes to transduce a variety of tissue and cell types.[33] Furthermore, transduction is potentially long lasting, directing gene expression over a period of months to years in non-dividing tissues.[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 (CD4BS) BnAb, b12, into mouse muscle.[35] More recently, similar delivery of further engineered self-complementary AAV (scAAV) vector encoding b12 IgG in humanized mice provided protection from infection when challenged intravenously or intravaginally with HIV-1.[29, 36] In addition, we have previously demonstrated 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.[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 due 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[38] and their progenitors[39, 40] as well as other stem cell types[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.[44, 45] In the present study, we evaluated multiple AAV serotypes for gene transfer to freshly immortalized endo- and ecto-cervical and vaginal epithelial cell lines derived from female Rh and Rh PGECs. We performed pilot AAVGFP 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 *in vitro*[46] and protects macaques against vaginal challenge with SHIV162p4 following Ab application to the cervical-vaginal mucosa[46] Our results show that intra-vaginal 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 potential for preventing HIV-1 infection in women during heterosexual transmission.

RESULTS

Generation of Rhesus macaque primary genital epithelial cell lines

Papillomavirus-immortalized cell lines from normal human vaginal, ecto- and endo-cervical cells have been previously generated and cultured for *in vitro* studies.[47, 48] These cell lines maintain expression of tissue-specific differentiation proteins and were similar to primary organotypic cultures.[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, ecto- and endo-cervical epithelia, respectively using retroviral vector LXSN-16E6E7 transduction.[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 (panel a). In Ca2+-supplemented (0.4 mM $CaCl_2$) keratinocyte serum-free medium, the immortalized cells formed tight colonies of attached sister cells (panels c-d) and the doubling time of the cultures was approximately 72 hrs. The Rh immortalized vaginal epithelial cell line Rh/V/E6E7 closely resembled the corresponding human immortalized vaginal cell line hu/V/E6E7 (Figure 1A, panels e-f). The immortalized cell lines were then stained with monoclonal antibodies specific for epithelial cell markers including, cytokeratin (ck) 19, 10 and 18 as well as secretory component (SC). As summarized in Table 1, Rh/V/E6E7, Rh/Ect/E6E7 and Rh/End/E6E7 cell lines stained positive for expression of ck19. In contrast, the Rh/End/E6E7 cell line did not stain positive for ck10 and Rh/V/E6E7 cell line was negative for ck18 staining. Thus, positive staining for ck19 and differential negative staining for ck10 and ck18 could be used to distinguish the three cell lines. Figure 1B shows representative positive staining of the immortalized vaginal epithelial cell line Rh/V/E6E7 for ck19 and ck10.

Identification of the optimal AAV serotype for transduction of Rh macaque female genital epithelial cell lines

Due to the fact that different AAV serotypes differentially transduce a wide variety of cells and tissues, 8 different AAV serotypes were evaluated to determine the optimal serotype(s) that most efficiently transduced female Rh genital epithelial cells. The transduction efficiencies of GFP-expressing AAV-1, 2, 3, 4, 5, 6, 8 and 9 (at multiplicity of infection (MOI) of 2×10^5 viral genome (vg) per cell) were evaluated on the three immortalized Rh cell lines. Expression of GFP protein was detected by flow cytometry and was represented as percentage of GFP positive cells (Figure 2A). GFP expression was also assessed visually by fluorescence microscopy (Figure 2B). All cell lines were successfully transduced with AAV-1, AAV-2, AAV-5 and AAV-6, with the most efficient transduction occurring with AAV-2 and AAV-6. In contrast, transduction efficiencies of serotypes AAV-3, AAV-4, AAV-8 and AAV-9 were low in these cell lines. To confirm that the inability of AAV-8 and AAV-9 to transduce Rh macaque female genital cell lines was due to their tropism for the specific cell type, and not to a defect in the vectors themselves, COS-1 cells were tested for transduction with AAV-8-GFP and AAV-9-GFP. Both vectors were able to transduce COS-1 cells effectively indicating that both vectors were functional in permissive cell types[37] and data not shown.

Anti-HIV b12 minibodies inhibit transfer of SHIV162p4 virus and its activity across the Rh macaque genital epithelial monolayer

We have previously reported the ability of anti-HIV-1 b12 minibodies to inhibit HIV-1 virus migration and infectivity using the human organotypic EpiVaginal™ tissue VEC model.[37] However, a similar model for non-human primates does not currently exist. Accordingly, we adapted the procedure developed by Bobardt *et al*[49] to examine the effects of the b12 minibodies on SHIV162p4 transfer through a monolayer of Rh PGECs (vaginal) in a transwell system. Anti-HIV-1 b12 minibodies were produced by transfecting 293 T cells with the AAV gene transfer vector pTR-b12scFvFc. SHIV162p4 virus (5 ng) with or without purified b12 scFvFc minibodies (10 ug) was then applied to the apical surface of the Rh monolayer. Following 3 hour (hr), 6 hr and overnight incubations, basal medium was collected and tested for the presence of SHIV162p4 viral particles using ELISA and infectivity assays. As shown in Figure 3, SHIV162p4 virus was effectively capable of penetrating the transwell system in the absence of an HIV-1-specific Ab as measured by p27 ELISA (Figure 3A). By contrast, in the presence of either b12 minibody or full-length b12 IgG1, SHIV162p4 transfer and infection was inhibited (Figure 3 A,B). Compared to untreated control, the inhibition of migration was 80%, 87% and 92% at 3hr, 6hr and overnight, respectively. This inhibition of migration (Figure 3A) was statistically significant at each time point ($P < 0.001$). In addition, compared to untreated control viral infectivity was inhibited by 96% and 80% in the samples collected after 3hr and 6hr respectively in the presence of b12 minibody ($p<0.0001$) (Figure 3B). The inhibition by b12 minibody and b12 IgG was comparable (Pearson chi^2 p=0.919 and 0.306 fat 3 hr and 6 hr, respectively). Similar results were obtained with endocervical and ectocervical monolayers in the transwell system (data not shown). These results are in agreement with published data on the ability of b12 to neutralize SHIV162p4[46]. These findings demonstrate that b12 minibodies are comparable to full-length b12 IgG1 in their ability to inhibit SHIV162p4 transfer through vaginal epithelial cells.

In vitro transduction of Rh macaque genital epithelial monolayer by AAV-6-b12 inhibits SHIV162p4 transfer

Monolayers of female Rh PGECs (vaginal) were transduced by using AAV-6 vectors encoding the anti-HIV-1 b12 minibody or an irrelevant control minibody (5×10^{10} particles in 100 µl and at MOI of 2×10^5 vg/cell) to the apical surface followed by SHIV162p4 virus (5 ng in 100 µl) at 4 days post 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 3hr and 6hrs 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 non-infectious [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 to the no treatment controls ($P< 0.001$ for each time point). In addition, supernatants from AAB-6-b12 transduced cells compared to control minibody transduced cells also showed 79%, 81% and 83% inhibition of migration at 3hr, 6hr and overnight, compared to 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 primary genital epithelial cells 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 $\times 10^{12}$ gc/animal (the approximate estimation of the MOI is 1×10^5 per cell) in 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 since 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 prior to transduction and at one, four, and eight weeks post 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 positive stem cell in the basal layer (Figure 5). No GFP positive cells were observed in the biopsy sections from 4 or 8 weeks post transduction; however, only two 1mm × 1mm 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/animal) was investigated in two animals. Immediately prior to application, the superficial epithelial cervical and vaginal mucosal layers were disrupted with a standard Pap smear cervical brush in order 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 post transduction were recovered and b12 minibodies, which were detectable at concentrations ranging between 450 pg to 800 pg/ml (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 minobodies 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, ecto- and endocervical cell lines and determined that AAV serotypes 2 and 6 allowed the highest level of transduction efficiency. We established Rh PGEC monolayer cultures and demonstrated *in vitro* inhibition of SHIV162p4 migration and infectivity using AAV-6-b12 minibody vector, a necessary step to block virus infection of susceptible immune cells present in the lamina propria of the vaginal and cervical mucosa.[2]

We also conducted proof-of-principle transduction studies on female Rh and demonstrated sustained detection of b12 minbodies in vaginal secretions following pre-conditioning with depoprovera and topical application of the AAV6-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 [50] In two Rh, AAV6-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 quantitatively assess 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 mini-endoscopic imaging.[51] Indeed, the latter technique was used to follow GFP/RFP expression in mice over seven days following intravaginal transduction with papilloma GFP pseudoviruses, although the stem cell compartment was not examined.[51, 52] In addition, 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 potentially gain easier access to the basal epithelial layers that contains p63+ stem cells, the mucosal surface was lightly scarified prior to AAV-6-b12 transduction using a standard Pap smear brush. A single application of 0.5×10^{12} gc/animal AAV-6-b12 resulted in a peak of 600-800 ng/ml b12 minibody expression in the vaginal secretions at 7 days post-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[29] and/or enhancements in delivery with formulations that are known to improve expression and transduction efficiency[53, 54] as well as the possible use of tyrosine-modified rAAV vectors[55-57] and directed evolution to increase stem cell gene transfer efficiencies[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 peri-coital microbicide applications.

We anticipate application of higher doses such as 10^{13} or 10^{14} gc/animal may yield a much higher concentration of b12 minibodies 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 $TCID_{50}$). 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 [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 CD4BS or other potent neutralization epitopes[27-29]. While previous microbicide studies with female Rh[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[60]. Therefore, future studies should evaluate combinations of the newly reported potent BnAbs antibodies[28, 61] as well as the use of IgA isotype to achieve a wider protection against HIV-1 isolates[60].

Despite the positive results of using AAV vectors in a number of preclinical and clinical settings, the preexisting 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 [62-65]. In our approach, we used AAV-6 vectors which were found to be resistant to the neutralizing effects of anti-AAV2 antibodies [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 anti-AAV antibodies did not correlate with elicitation of antibodies to the transgene [34, 67, 68]. Readministration of AAV2/9 in the presence of high levels of circulating neutralizing antibodies also had minimal effect on transgene expression [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 [69-71]. Another safety concern is based on detection of AAV DNA in human genital tissues and in material from spontaneous abortions [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 [74].

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[15, 75-78] but 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.[15] 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,[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 need to be evaluated fully. Accordingly, our data provide justification for moving this approach towards 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 secretion of protective levels of neutralizing b12 antibody (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.

MATERIALS AND METHODS

Growth media

Rh macaque female genital epithelial cells were cultured in keratinocyte serum-free medium (GIBCO/BRL Life Technologies, Grand Island, NY), supplemented with bovine pituitary extract and recombinant human epidermal growth factor. The medium was further supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and $CaC1_2$ to a final calcium concentration of 0.4 mM. All other cell lines used in this study were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA).

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), is a CXCR4-positive HeLa cell line that expresses CD4 and HIV-1 co-receptor CCR5; it also contains integrated reporter genes for luciferase and *E. coli* beta-galactosidase, under the control of an HIV long-terminal repeat sequence (*tat* gene) which allows for quantification of HIV infection. PA317, 293T and COS-1 cells were purchased from ATCC. All cells and cultures were maintained at 37°C in a 5% CO_2 humidified incubator. The R-tropic SHIV-162p4 virus is based on molecular clones of SIVmac239 and the R5 HIV-1 primary isolate SF162 (derived from *in vivo after three* serial passages in Rh macaques[79] was a gift from Dr. Cheng-Mayer C. (Aaron Diamond AIDS Research Center, NY).

Establishing Rh macaque primary genital epithelial cell 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 IACCUC regulations. Tissues were collected in cold Hanks' Balanced Salt Solution without Ca2+ and Mg 2+ and supplemented with penicillin (100 U/ml), streptomycin (100 ug/ml), and gentamicin (50 ug/ml) (GIBCO); and epithelial cells were isolated using a modified previously described protocol.[48] Briefly, tissue was minced into very small pieces and then digested for 3h at 37°C in 1mg/ml of collagenase-dispase containing 1mg/ml of DNase (Sigma), with gentle stirring; the mixture was then passed through a cell strainer (250-μm), spun down (1500 rpm) for 20 min and re-suspended in DMEM with 10 % fetal calf serum (FCS). After additional centrifugation, the pellet was re-suspended in epithelial cell selection medium (Ker-sfm) inT-25 flasks; the cultures were fed every 3 days for the next 6-9 days, then sub-cultured to expand for cryopreservation and to set up cultures. Cells were passed twice prior to transduction with LXSN-16E6E7 retroviral vector packaged by the amphotropic fibroblast 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 tissue were cryopreserved in liquid nitrogen or at −80°C after the second passage by freezing in 90% calf serum (HyClone) and 10% sulfoxide dimethyl (DMSO; 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 AAV1 and AAV6 prior to inoculation, and were treated with once monthly subcutaneous Depo-Provera throughout the study period beginning 2 months prior to initial treatment. Animals were sedated using standard procedures, placed in ventral recumbency with hips elevated, followed without (AAV6-GFP) or with (AAV6-b12) pre-treatment by gentle abrasion of the vaginal and cervical mucosa with a Pap smear cervical brush (Kansas Pathology Consultants, Wichita, KS). No speculum was used to avoid loss of transduction fluid. For preparation of AAV6-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 0.5ml/animal of AAV-6-GFP or AAV-6-b12 (0.5×10^{12} genomic copies diluted in PBS) using a 1ml syringe that was inserted as deep as possible into the vagina. Hip elevation was maintained for 30 minutes 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 speculum-guided cervicovaginal biopsies were obtained at weeks -1 (pre-inoculation), 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) and Penn Vector Core, University of Pennsylvania, Philadelphia, PA) whereas AAV serotypes 3 and 4 expressing GFP were obtained from the AAV core at the University of North Carolina, Chapel Hill. AAV-6 expressing b12 minibody was obtained commercially (Virapur LLC, San Diego, CA).

Transduction of cells, flow cytometry analysis and fluorescence microscopy

For AAV transduction, 5×10^4 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^{10} genomic copies and at MOI of 2×10^5 vg/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) and was represented as percentage of GFP positive cells, and was also assessed visually by fluorescence microscopy.

Production of b12 minibodies and gp120 ELISA binding assay

To produce the b12 minibodies, 293T cells were transfected with pTR-b12scFvFc using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Sixteen hours after transfection, the media was replaced; cells were further incubated for another 48 h. The supernatant was harvested, sterile-filtered, and purified after overnight incubation at 4°C with Protein A agarose beads (GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions. The b12minibody minibodies proteins were eluted with IgG elution buffer (Thermo Scientific, Waltham, MA) and the buffer was exchanged in PBS using Amicon Ultra-15 centrifugal filtration units (30 kDa molecular weight cut-off; Millipore, New Bedford, MA). Concentration of the purified b12 minibodies was measured using a human IgG ELISA kit (Bethyl Laboratories, Montgomery, TX). Ninety-six-well microtiter plates were coated overnight at 4°C with 10 ng/well of HIV-1 bal gp120 (NIH-ARRRP, Germantown, MD. Cat. # 4961) in 0.05 M carbonate-bicarbonate buffer (pH 9.6, Sigma) and blocked in PBS (1% BSA) for 1 h; serial dilutions of b12 minibodies were added to the plate for 1 h at room temperature. After washing, HRP-conjugated, affinity-purified goat anti-human IgG (Bethyl Laboratories, Montgomery, TX) was added (1:50,000) for 1 h. After extensive washing, the plate was developed by addition of TMB substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and detected by reading the absorbance (OD) at 450 nm.

Preparation of monolayers of Rh macaque cells

Rh primary genital epithelial cells were seeded at a density of 10^5 cells/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 (TEER) (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 (70kDa).

SIV p27 antigen-capture ELISA assay

SHIV-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).

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/well) and grown overnight. The medium was then removed, and cells incubated with 100 μl of media collected at different time points (up to 24 h) from the lower chambers of the Rh macaque monolayer transwell cultures. After 48 h of incubation, the cells were washed and lysed. The luciferase activity was quantified with the luciferase assay system (Promega, Cat. #E1501) and measured using the Centro LB 960 Luminometer (Berthold, Bad Wildbad, Germany).

Meso Scale Discovery (MSD) Assay—This assay in principle is similar to an ELISA assay with the outcome measured with MSD technology, which is based on Electrochemiluminescence (ECL) 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 ul of HIV-1 gp120 bal protein at 40 ug/ml, and incubated overnight at 4°C. The following day, the antigen-coated plate was incubated at 37°C for 1 h with 2% BSA 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 1h incubation at 37°C, plates were washed with 0.05% PBS-T; and 25 μl (500ug/ml) of Sulfo-Tag-labeled goat anti-human IgG secondary antibody (Meso Scale Discovery, MD, USA Cat. # R32AJ-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 a MSD sector imager, Model no. 2400.

Immunofluorescence and Immunochemistry

Immunofluorescence analysis was performed on Rh Ecto-, Endo-, and Vaginal immortalized cell lines by growing them for 3 days on 13 mm Thermanox coverslips in 24-well Falcon tissue culture plates with keratinocyte serum-free medium (ker-sfm; GIBCO) (Nunc, Naperville, IL) (Becton-Dickinson, Rutherford, NJ). Cells on coverslips were fixed with cold absolute methanol for 5 minutes and quickly rinsed with distilled water. The cell lines were then phenotyped using the specific epithelial cell markers, ck19, ck18, ck10 and secretory component (SC, polyIgA receptor) monoclonal antibodies. Cells were then examined under fluorescence microscope. *Immunochemistry*; Vaginal and cervical biopsies from normal Rh or Rh intra-vaginally transduced with AAV-6-GFP were fixed in 2% paraformaldehyde for 2 hours before being cryopreserved in 30% sucrose, embedded in Tissue-Tek cryo O.C.T compound (Thermo Scientific), frozen in 2-methylbutane (Fisher), and stored at −80°C. Blocks were cryosectioned at 5 microns 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 # sc-8431, Santa Cruz, CA) at room temperature for 30 minutes followed by biotinylated goat anti-mouse IgG (1:200 Cat # BA9200, Vector Burlingame CA) and streptavidin 488 (1:500, Cat. # S-11223, Life Technology, Grand Island NY) for 30 minutes each. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) in the mounting media. Controls included isotype matched irrelevant antibodies. Tissues were then examined by fluorescence microscopy for GFP expression, using a Leica SP5 Inverted Laser Scanning Confocal Microscope (Leica Microsystems, Buffalo Grove, IL) with further image processing using ImageJ software (National Institutes of Health, Bethesda, Maryland).

Statistical Analysis

All statistical evaluations were performed using Two-sample t-test. $p<0.01$ was considered statistically significant.

Acknowledgments

We thank Dennis Burton (The Scripps Research Institute, La Jolla, CA) for providing the scFv b12DNA plasmid, and Leonidas Stamatatos for providing the SHIV-162p4 virus. We thank clinical staff Joshua Kramer, Amber Hoggatt and Matt Beck for veterinary services and Karen Boisvert for microscopy and immunofluorescence assistance. This work was supported by NIH R21/R33 AI079767 to W.A.M, by the New England Primate Research Center Base grant P51OD011103-51 (NEPRC) and the T32 training grant T32OD011064 (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 and behavior. 2011; 15(4):761–6. [PubMed: 20703793]
2. Hladik F, Hope TJ. HIV infection of the genital mucosa in women. Current HIV/AIDS reports. 2009; 6(1):20–8. [PubMed: 19149993]
3. Carias A, McCoombe S, McRaven M, Anderson M, Galloway N, Vandergrift N, et al. Defining the Interaction of HIV-1 with the Mucosal Barriers of the Female Reproductive Tract. Journal of virology. 2013
4. Dezzutti CS, Uranker K, Bunge KE, Richardson-Harman N, Macio I, Hillier SL. HIV-1 Infection of Female Genital Tract Tissue for use in Prevention Studies: Short title: Ex vivo challenge using female tissue. J Acquir Immune Defic Syndr. 2013
5. Haase AT. Early events in sexual transmission of HIV and SIV and opportunities for interventions. Annual review of medicine. 2011; 62:127–39.
6. Hladik F, McElrath MJ. Setting the stage: host invasion by HIV. Nature reviews. Immunology. 2008; 8(6):447–57.
7. Cole AM, Cole AL. Antimicrobial polypeptides are key anti-HIV-1 effector molecules of cervicovaginal host defense. Am J Reprod Immunol. 2008; 59(1):27–34. [PubMed: 18154593]
8. Ghosh M, Fahey JV, Shen Z, Lahey T, Cu-Uvin S, Wu Z, et al. Anti-HIV activity in cervical-vaginal secretions from HIV-positive and -negative women correlate with innate antimicrobial levels and IgG antibodies. PloS one. 2010; 5(6):e11366. [PubMed: 20614007]
9. Kaushic C, Ferreira VH, Kafka JK, Nazli A. HIV infection in the female genital tract: discrete influence of the local mucosal microenvironment. Am J Reprod Immunol. 2010; 63(6):566–75. [PubMed: 20384619]
10. King AE, Critchley HO, Kelly RW. Presence of secretory leukocyte protease inhibitor in human endometrium and first trimester decidua suggests an antibacterial protective role. Molecular human reproduction. 2000; 6(2):191–6. [PubMed: 10655462]
11. Novak RM, Donoval BA, Graham PJ, Boksa LA, Spear G, Hershow RC, et al. Cervicovaginal levels of lactoferrin, secretory leukocyte protease inhibitor, and RANTES and the effects of coexisting vaginoses in human immunodeficiency virus (HIV)-seronegative women with a high risk of heterosexual acquisition of HIV infection. Clinical and vaccine immunology : CVI. 2007; 14(9):1102–7. [PubMed: 17671228]
12. Shukair SA, Allen SA, Cianci GC, Stieh DJ, Anderson MR, Baig SM, et al. Human cervicovaginal mucus contains an activity that hinders HIV-1 movement. Mucosal immunology. 2013; 6(2):427–34. [PubMed: 22990624]
13. Hladik F, Sakchalathorn P, Ballweber L, Lentz G, Fialkow M, Eschenbach D, et al. Initial events in establishing vaginal entry and infection by human immunodeficiency virus type-1. Immunity. 2007; 26(2):257–70. [PubMed: 17306567]
14. Turville SG, Peretti S, Pope M. Lymphocyte-dendritic cell interactions and mucosal acquisition of SIV/HIV infection. Current opinion in HIV and AIDS. 2006; 1(1):3–9. [PubMed: 19372776]

15. 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(5996):1168–74. [PubMed: 20643915]
16. Feldblum PJ, Adeiga A, Bakare R, Wevill 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(1):e1474. [PubMed: 18213382]
17. Halpern V, Ogunsola F, Obunge O, Wang CH, Onyejepu N, Oduyebo 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(11):e3784. [PubMed: 19023429]
18. 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 301): a phase 3, randomised, double-blind, parallel-group trial. Lancet. 2010; 376(9749):1329–37. [PubMed: 20851460]
19. 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(12):e1312. [PubMed: 18091987]
20. Skoler-Karpoff S, Ramjee G, Ahmed K, Altini L, Plagianos 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(9654):1977–87. [PubMed: 19059048]
21. 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. The New England journal of medicine. 2008; 359(5):463–72. [PubMed: 18669425]
22. Van Damme L, Ramjee G, Alary M, Vuylsteke B, Chandeying 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(9338):971–7. [PubMed: 12383665]
23. 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(1):10–5. [PubMed: 21134498]
24. Hankins CA, Dybul MR. The promise of pre-exposure prophylaxis with antiretroviral drugs to prevent HIV transmission: a review. Current opinion in HIV and AIDS. 2013; 8(1):50–8. [PubMed: 23201856]
25. Dey B, Lagenaur LA, Lusso P. Protein-Based HIV-1 Microbicides. Current HIV research. 2013; 11(7):576–94. [PubMed: 24382025]
26. Masse BR, Boily MC, Dimitrov D, Desai K. Efficacy dilution in randomized placebo-controlled vaginal microbicide trials. Emerging themes in epidemiology. 2009; 6:5. [PubMed: 19818138]
27. 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. Proceedings of the National Academy of Sciences of the United States of America. 2013; 110(33):13540–5. [PubMed: 23878231]
28. Walker LM, Huber M, Doores KJ, Falkowska E, Pejchal R, Julien JP, et al. Broad neutralization coverage of HIV by multiple highly potent antibodies. Nature. 2011; 477(7365):466–70. [PubMed: 21849977]
29. Balazs AB, Chen J, Hong CM, Rao DS, Yang L, Baltimore D. Antibody-based protection against HIV infection by vectored immunoprophylaxis. Nature. 2012; 481(7379):81–4.
30. Clark KR. Recent advances in recombinant adeno-associated virus vector production. Kidney international. 2002; 61(1 Suppl):S9–15. [PubMed: 11841606]
31. Johnson PR, Schnepp 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. Nature medicine. 2009; 15(8):901–6.
32. Schultz BR, Chamberlain JS. Recombinant adeno-associated virus transduction and integration. Molecular therapy : the journal of the American Society of Gene Therapy. 2008; 16(7):1189–99. [PubMed: 18500252]

33. Xiao PJ, Lentz TB, Samulski RJ. Recombinant adeno-associated virus: clinical application and development as a gene-therapy vector. Therapeutic delivery. 2012; 3(7):835–56. [PubMed: 22900466]
34. 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 rAAV1-AAT gene therapy. Proceedings of the National Academy of Sciences of the United States of America. 2009; 106(38): 16363–8. [PubMed: 19706466]
35. Lewis AD, Chen R, Montefiori DC, Johnson PR, Clark KR. Generation of neutralizing activity against human immunodeficiency virus type 1 in serum by antibody gene transfer. Journal of virology. 2002; 76(17):8769–75. [PubMed: 12163597]
36. Balazs AB, Ouyang Y, Hong CM, Chen J, Nguyen SM, Rao DS, et al. Vectored immunoprophylaxis protects humanized mice from mucosal HIV transmission. Nature medicine. 2014; 20(3):296–300.
37. Abdel-Motal UM, Sarkis PT, Han T, Pudney J, Anderson DJ, Zhu Q, et al. Anti-gp120 minibody gene transfer to female genital epithelial cells protects against HIV-1 virus challenge in vitro. PloS one. 2011; 6(10):e26473. [PubMed: 22031835]
38. Yan Z, Lei-Butters DC, Keiser NW, Engelhardt JF. Distinct transduction difference between adeno-associated virus type 1 and type 6 vectors in human polarized airway epithelia. Gene therapy. 2013; 20(3):328–37. [PubMed: 22695783]
39. Limberis MP, Adam VS, Wong G, Gren J, Kobasa D, Ross TM, et al. Intranasal antibody gene transfer in mice and ferrets elicits broad protection against pandemic influenza. Science translational medicine. 2013; 5(187):187ra72.
40. Limberis MP, Wilson JM. Adeno-associated virus serotype 9 vectors transduce murine alveolar and nasal epithelia and can be readministered. Proceedings of the National Academy of Sciences of the United States of America. 2006; 103(35):12993–8. [PubMed: 16938846]
41. Asuri P, Bartel MA, Vazin T, Jang JH, Wong TB, Schaffer DV. Directed evolution of adeno-associated virus for enhanced gene delivery and gene targeting in human pluripotent stem cells. Molecular therapy : the journal of the American Society of Gene Therapy. 2012; 20(2):329–38. [PubMed: 22108859]
42. Locke M, Ussher JE, Mistry R, Taylor JA, Dunbar PR. Transduction of human adipose-derived mesenchymal stem cells by recombinant adeno-associated virus vectors. Tissue engineering. Part C, Methods. 2011; 17(9):949–59. [PubMed: 21563982]
43. Veldwijk MR, Sellner L, Stiefelhagen M, Kleinschmidt JA, Laufs S, Topaly J, et al. Pseudotyped recombinant adeno-associated viral vectors mediate efficient gene transfer into primary human CD34(+) peripheral blood progenitor cells. Cytotherapy. 2010; 12(1):107–12. [PubMed: 19929455]
44. Joag SV. Primate models of AIDS. Microbes and infection / Institut Pasteur. 2000; 2(2):223–9.
45. Lackner AA, Veazey RS. Current concepts in AIDS pathogenesis: insights from the SIV/macaque model. Annual review of medicine. 2007; 58:461–76.
46. Parren PW, Marx PA, Hessell AJ, Luckay A, Harouse J, Cheng-Mayer C, et al. Antibody protects macaques against vaginal challenge with a pathogenic R5 simian/human immunodeficiency virus at serum levels giving complete neutralization in vitro. Journal of virology. 2001; 75(17):8340–7. [PubMed: 11483779]
47. Fichorova RN, Anderson DJ. Differential expression of immunobiological mediators by immortalized human cervical and vaginal epithelial cells. Biology of reproduction. 1999; 60(2): 508–14. [PubMed: 9916021]
48. Fichorova RN, Rheinwald JG, Anderson DJ. Generation of papillomavirus-immortalized cell lines from normal human ectocervical, endocervical, and vaginal epithelium that maintain expression of tissue-specific differentiation proteins. Biology of reproduction. 1997; 57(4):847–55. [PubMed: 9314589]
49. Bobardt MD, Chatterji U, Selvarajah S, Van der Schueren B, David G, Kahn B, et al. Cell-free human immunodeficiency virus type 1 transcytosis through primary genital epithelial cells. Journal of virology. 2007; 81(1):395–405. [PubMed: 17050597]

50. Veazey RS, Shattock RJ, Pope M, Kirijan JC, Jones J, Hu Q, et al. Prevention of virus transmission to macaque monkeys by a vaginally applied monoclonal antibody to HIV-1 gp120. Nature medicine. 2003; 9(3):343–6.
51. Mitsunaga M, Kosaka N, Kines RC, Roberts JN, Lowy DR, Schiller JT, et al. In vivo longitudinal imaging of experimental human papillomavirus infection in mice with a multicolor fluorescence mini-endoscopy system. Cancer Prev Res (Phila). 2011; 4(5):767–73. [PubMed: 21430072]
52. Gordon SN, Kines RC, Kutsyna G, Ma ZM, Hryniewicz A, Roberts JN, et al. Targeting the vaginal mucosa with human papillomavirus pseudovirion vaccines delivering simian immunodeficiency virus DNA. J Immunol. 2012; 188(2):714–23. [PubMed: 22174446]
53. Ellis BL, Hirsch ML, Porter SN, Samulski RJ, Porteus MH. Zinc-finger nuclease-mediated gene correction using single AAV vector transduction and enhancement by Food and Drug Administration-approved drugs. Gene therapy. 2013; 20(1):35–42. [PubMed: 22257934]
54. Wheeler LA, Vrbanac V, Trifonova R, Brehm MA, Gilboa-Geffen A, Tanno S, et al. Durable Knockdown and Protection From HIV Transmission in Humanized Mice Treated With Gel-formulated CD4 Aptamer-siRNA Chimeras. Molecular therapy : the journal of the American Society of Gene Therapy. 2013; 21(7):1378–89. [PubMed: 23629001]
55. Jang JH, Koerber JT, Kim JS, Asuri P, Vazin T, Bartel M, et al. An evolved adeno-associated viral variant enhances gene delivery and gene targeting in neural stem cells. Molecular therapy : the journal of the American Society of Gene Therapy. 2011; 19(4):667–75. [PubMed: 21224831]
56. Kauss MA, Smith LJ, Zhong L, Srivastava A, Wong KK Jr. Chatterjee S. Enhanced long-term transduction and multilineage engraftment of human hematopoietic stem cells transduced with tyrosine-modified recombinant adeno-associated virus serotype 2. Human gene therapy. 2010; 21(9):1129–36. [PubMed: 20486772]
57. Li M, Jayandharan GR, Li B, Ling C, Ma W, Srivastava A, et al. High-efficiency transduction of fibroblasts and mesenchymal stem cells by tyrosine-mutant AAV2 vectors for their potential use in cellular therapy. Human gene therapy. 2010; 21(11):1527–43. [PubMed: 20507237]
58. Bartel MA, Weinstein JR, Schaffer DV. Directed evolution of novel adeno-associated viruses for therapeutic gene delivery. Gene therapy. 2012; 19(6):694–700. [PubMed: 22402323]
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. Nature medicine. 2009; 15(8):951–4.
60. Hur EM, 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(23):4571–82. [PubMed: 23065154]
61. Klein F, Halper-Stromberg A, Horwitz JA, Gruell H, Scheid JF, Bournazos S, et al. HIV therapy by a combination of broadly neutralizing antibodies in humanized mice. Nature. 2012; 492(7427): 118–22. [PubMed: 23103874]
62. Boutin S, Monteilhet 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. Human gene therapy. 2010; 21(6):704–12. [PubMed: 20095819]
63. Calcedo R, Vandenberghe LH, Gao G, Lin J, Wilson JM. Worldwide epidemiology of neutralizing antibodies to adeno-associated viruses. The Journal of infectious diseases. 2009; 199(3):381–90. [PubMed: 19133809]
64. Louis Jeune V, Joergensen JA, Hajjar RJ, Weber T. Pre-existing anti-adeno-associated virus antibodies as a challenge in AAV gene therapy. Human gene therapy methods. 2013; 24(2):59–67. [PubMed: 23442094]
65. Monteilhet V, Saheb 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. Molecular therapy : the journal of the American Society of Gene Therapy. 2011; 19(11):2084–91. [PubMed: 21629225]
66. Rutledge EA, Halbert CL, Russell DW. Infectious clones and vectors derived from adeno-associated virus (AAV) serotypes other than AAV type 2. Journal of virology. 1998; 72(1):309–19. [PubMed: 9420229]

67. Manno CS, Chew AJ, 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(8): 2963–72. [PubMed: 12515715]
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. Arteriosclerosis, thrombosis, and vascular biology. 2008; 28(12):2303–4.
69. Hareendran S, Balakrishnan B, Sen D, Kumar S, Srivastava A, Jayandharan GR. Adeno-associated virus (AAV) vectors in gene therapy: immune challenges and strategies to circumvent them. Reviews in medical virology. 2013; 23(6):399–413. [PubMed: 24023004]
70. Mingozzi F, Anguela XM, Pavani G, Chen Y, Davidson RJ, Hui DJ, et al. Overcoming preexisting humoral immunity to AAV using capsid decoys. Science translational medicine. 2013; 5(194): 194ra92.
71. Zhu J, Huang X, Yang Y. The TLR9-MyD88 pathway is critical for adaptive immune responses to adeno-associated virus gene therapy vectors in mice. The Journal of clinical investigation. 2009; 119(8):2388–98. [PubMed: 19587448]
72. Malhomme O, Dutheil N, Rabreau M, Armbruster-Moraes E, Schlehofer JR, Dupressoir T. Human genital tissues containing DNA of adeno-associated virus lack DNA sequences of the helper viruses adenovirus, herpes simplex virus or cytomegalovirus but frequently contain human papillomavirus DNA. The Journal of general virology. 1997; 78(Pt 8):1957–62. [PubMed: 9266994]
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 and in material from spontaneous abortion. Journal of medical virology. 1994; 44(2):215–22. [PubMed: 7852963]
74. Schlehofer JR, Boeke C, Reuland M, Eggert-Kruse W. Presence of DNA of adeno-associated virus in subfertile couples, but no association with fertility factors. Hum Reprod. 2012; 27(3):770–8. [PubMed: 22215624]
75. Dereuddre-Bosquet N, Morellato-Castillo L, Brouwers J, Augustijns P, Bouchemal K, Ponchel G, et al. MiniCD4 microbicide prevents HIV infection of human mucosal explants and vaginal transmission of SHIV(162P3) in cynomolgus macaques. PLoS pathogens. 2012; 8(12):e1003071. [PubMed: 23236282]
76. Veselinovic M, Neff CP, Mulder LR, Akkina R. Topical gel formulation of broadly neutralizing anti-HIV-1 monoclonal antibody VRC01 confers protection against HIV-1 vaginal challenge in a humanized mouse model. Virology. 2012; 432(2):505–10. [PubMed: 22832125]
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. Antimicrobial agents and chemotherapy. 2010; 54(3):1343–6. [PubMed: 20086159]
78. Yamamoto HS, Xu Q, Fichorova RN. Homeostatic properties of Lactobacillus jensenii engineered as a live vaginal anti-HIV microbicide. BMC microbiology. 2013; 13:4. [PubMed: 23298379]
79. Harouse JM, Gettie A, Tan RC, Blanchard J, Cheng-Mayer C. Distinct pathogenic sequela in rhesus macaques infected with CCR5 or CXCR4 utilizing SHIVs. Science. 1999; 284(5415):816–9. [PubMed: 10221916]

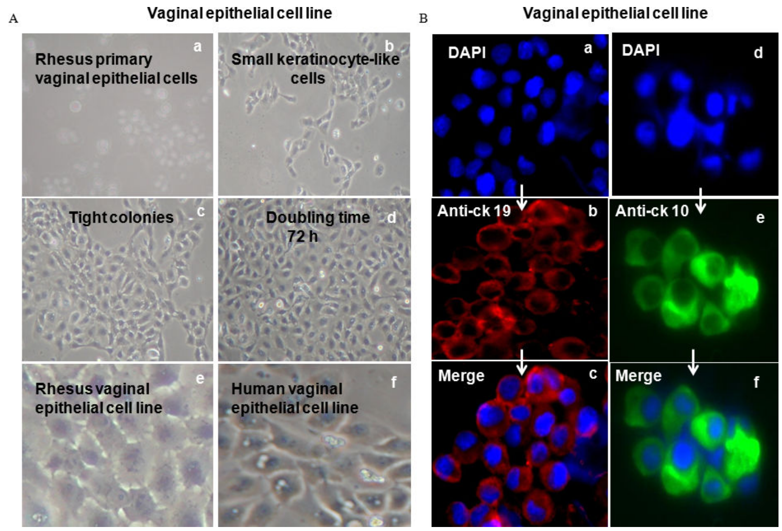

Fig.1.

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 Ca2+-supplemented keratinocyte serum-free medium, the immortalized cells formed tight colonies of attached cells (c-d). Doubling time of the cultures was approximately 72 h (c-d). Note that both Rh macaque (e) and human vaginal epithelial cell line (f) at the light microscopic level have a similar morphology.

Fig.1B. 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,d) DAPI staining, (c) showing (a) and (b) combined and (f) showing (d) and (e) combined.

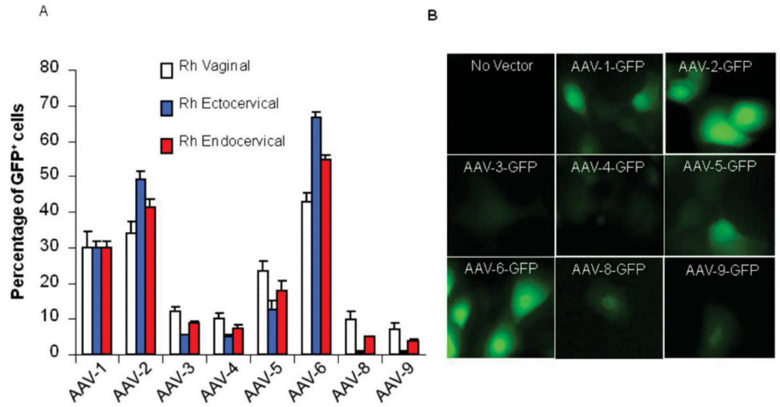

Fig.2.

A-B. Transduction of Rh macaque endocervical, ectocervical and vaginal epithelial cells by various AAV serotypes expressing GFPat MOI of 2×10^5 vg/cell. (A) Expression of GFP protein by transduced cells was detected by FACS, and presented as percentages of GFP positive cells. Note that AAV-2 and AAV-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.

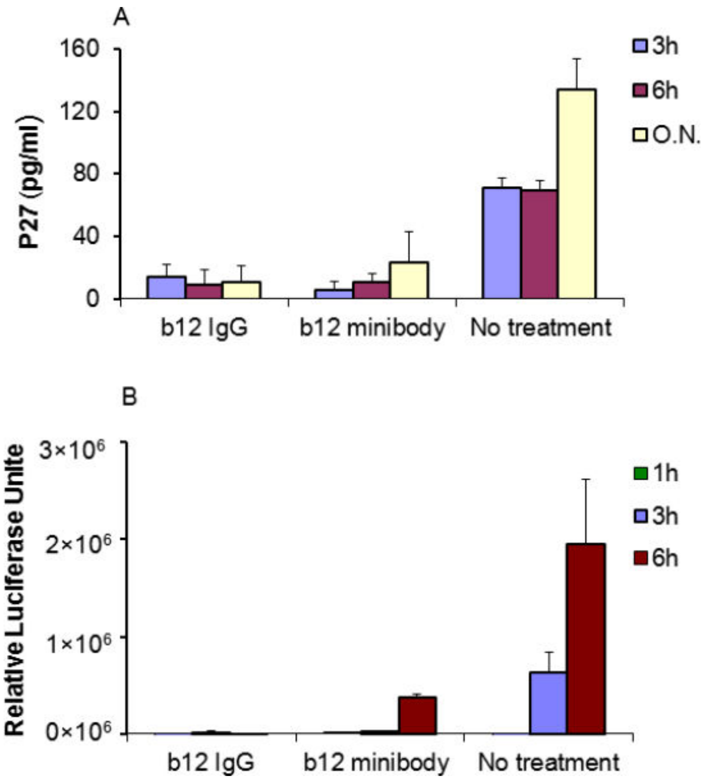

Fig.3.

A-B. Inhibition of SHIV162p4 virus transfer and infectivity by b12 minibodies across a monolayer of Rh PGECs (vaginal). After pre-incubating b12 minibodies or full-length b12 IgG (10 ug/ml) with SHIV162p4 (30 ng) for 1 hour, medium from the basal chambers was collected at different time points; inhibition of SHIV162p4 transfer was measured by p27 ELISA to measure p27 content (A) and inhibition of virus infectivity was evaluated by incubating on TZM-bl target cells (B). Note that media collected at 3 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.

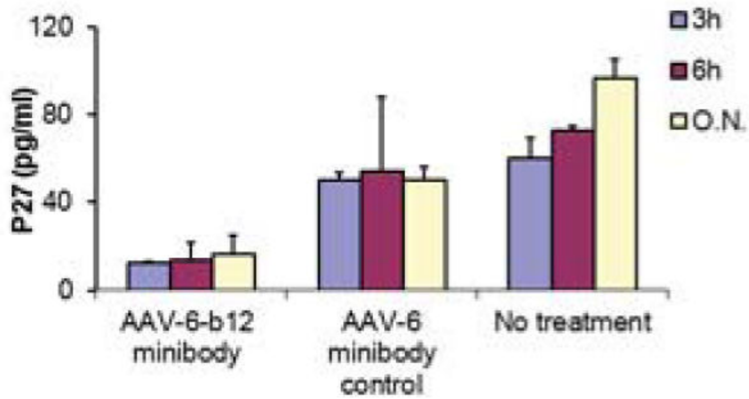

Fig.4.

In vitro SHIV162p4 virus challenge. AAV-6-b12 minibody gene, or irrelevant minibody gene control at 5×10^{10} particles were applied to the apical surface of Rh macaque genital epithelial monolayers for transduction. Four days later, SHIV162p4 (5 ng) was applied on the apical surface of the monolayer. Medium from the basal chambers was collected at various time points and tested for inhibition of viral transfer with use of ELISA to measure p27 content. Note that media collected from basal chambers treated with SHIV162p4 plus b12 minibodies had many fewer SHIV162p4 virus particles compared to the untreated samples.

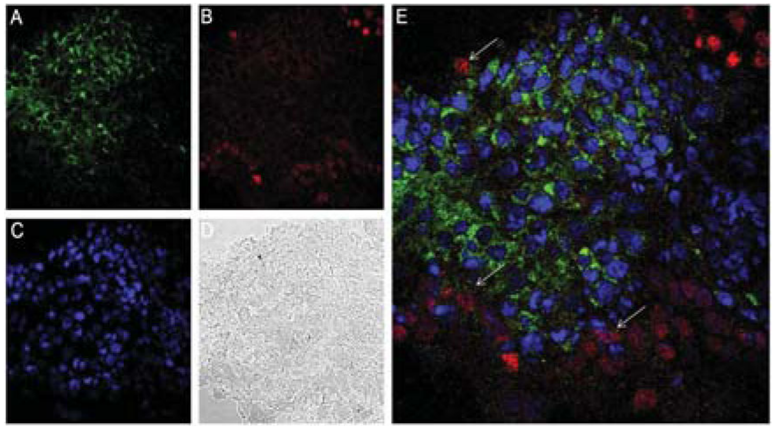

Fig.5.

In vivo transduction of Rh macaque female genital tract with AAV-6-GFP. Fluorescence microscopy was used to examine cervical biopsy sections. A mucosal biopsy tissue sample with GFP transduced cells (green, A and E) taken one week after transduction with AAV-6-GFP is shown; p63 immunofluorescence staining (red, B and E) highlights the basal epithelial cell layer. A transduced basal cell is indicated (arrow, panel E). Nuclei are stained with DAPI (blue, C and E). Differential interference contrast (DIC) (D). Main images at 40×, insets at 80×.

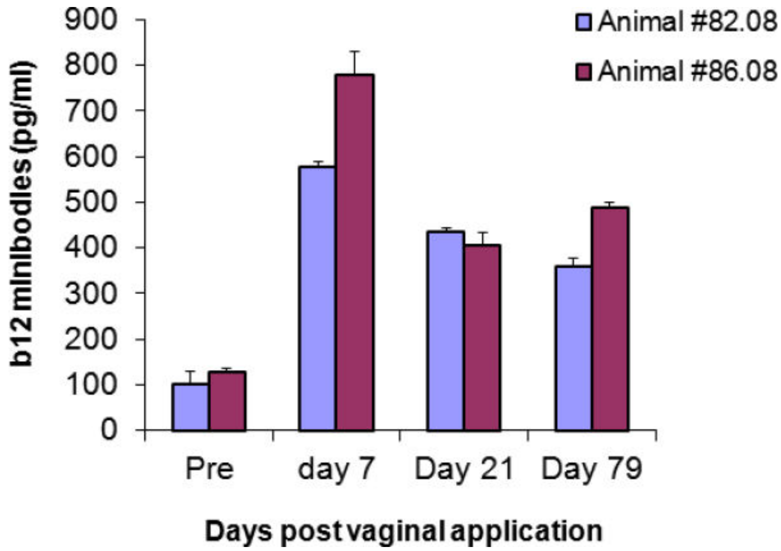

Fig. 6.

Concentration of b12 minibodies in vaginal secretion subsequent to *in vivo* transduction with AAV-6-b12. Vaginal secretions from each animal were absorbed to cellulose wicks. Samples were taken at different time points after AAV-6-b12 transduction. The concentration of b12 minibodies in vaginal secretion was determined by MSD assay from the clarified supernatant extracted from the wicks.

Table 1

Rh macaque genital epithelial cell lines stained with various cytokeratin-specific antibodies.

Protein	Vaginal epithelial	Ectocervical	Endocervical
ck 19	+	+	+
ck 18	-	+	+
ck 10	+	+	-
SC (polyIgA receptor)	(+)	-	+

- no expression, (+) weak expression and + positive expression