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ORIGINAL ARTICLE

Prolonged expression of an anti-HIV-1 gp120 minibody to the female rhesus macaque lower genital tract by AAV gene transfer

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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 green fluorescent protein (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 intravaginally, including p63+ epithelial stem cells. Moreover, intravaginal 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.

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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 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,[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] 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.[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 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 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,

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transduction is potentially long lasting, directing gene expression over a period of months to years in nondividing 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 BnAb, b12, into mouse muscle.[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.[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.[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[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 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 *in vitro*[46] and protects macaques against vaginal challenge with SHIV162p4 following Ab application to the cervical–vaginal mucosa.[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 *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, ectocervical and endocervical 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

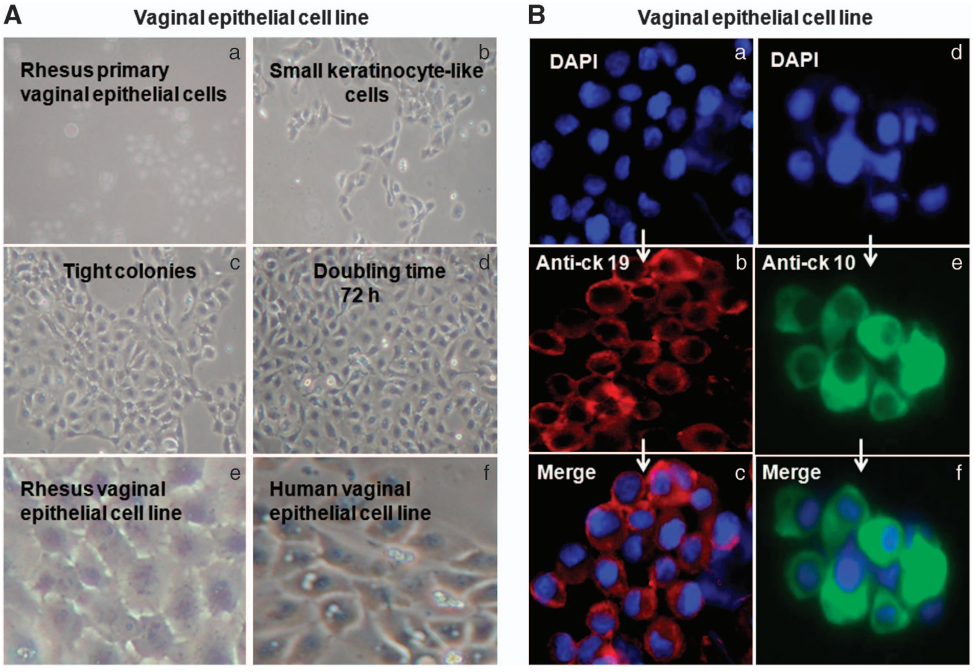

Figure 1. (**A**) Light microscopic examination of the Rh macaque vaginal epithelial cell line Rh/V/E6E7 reveals small keratinocyte-like cells (b), in contrast to the primary cell cultures (a). In Ca^{2+}-supplemented keratinocyte serum-free medium, the immortalized cells formed tight colonies of attached 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.

cell cultures (panel a). In Ca^{2+}-supplemented (0.4 mM $CaCl_2$) keratinocyte serum-free medium, the immortalized cells formed tight colonies of attached sister cells (panels c and d) and the doubling time of the cultures was approximately 72 h. 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 and 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. As summarized in Table 1, Rh/V/E6E7, Rh/Ect/E6E7 and Rh/End/E6E7 cell lines stained positive for the 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

Owing to the fact that different AAV serotypes differentially transduce a wide variety of cells and tissues, eight 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 the percentage of GFP + cells (Figure 2a). GFP expression was also assessed visually by fluorescence microscopy (Figure 2b). All cell lines were successfully transduced with AAV-1, −2, −5 and −6, with the most efficient transduction occurring with AAV-2 and −6. In contrast, transduction efficiencies of serotypes AAV-3, −4, −8 and −9 were low in these cell lines. To confirm that the inability of AAV-8 and −9 to transduce Rh macaque female genital cell lines was owing 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 (Abdel-Motal[37] and data not shown).

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.

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 nonhuman 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-b12-scFvFc. SHIV162p4 virus (5 ng) with or without purified b12-scFvFc minibodies (10 μg) was then applied to the apical surface of the Rh monolayer. Following 3 h, 6 h and overnight incubations, basal medium was collected and tested for the presence of SHIV162p4 viral particles using enzyme-linked immunosorbent assay (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 (Figures 3a and b). Compared with untreated control, the inhibition of migration was 80%, 87% and 92% at 3 h, 6 h and overnight, respectively. This inhibition of migration (Figure 3a) was statistically significant at each time point ($P < 0.001$). In addition, compared with untreated control, viral infectivity was inhibited by 96% and 80% in the samples collected after 3 and 6 h, respectively, in the presence of b12 minibody ($P < 0.0001$) (Figure 3b). The inhibition by b12 minibody and b12 IgG was comparable (Pearson's χ^2 $P = 0.919$ and 0.306 at 3 and 6 h, 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

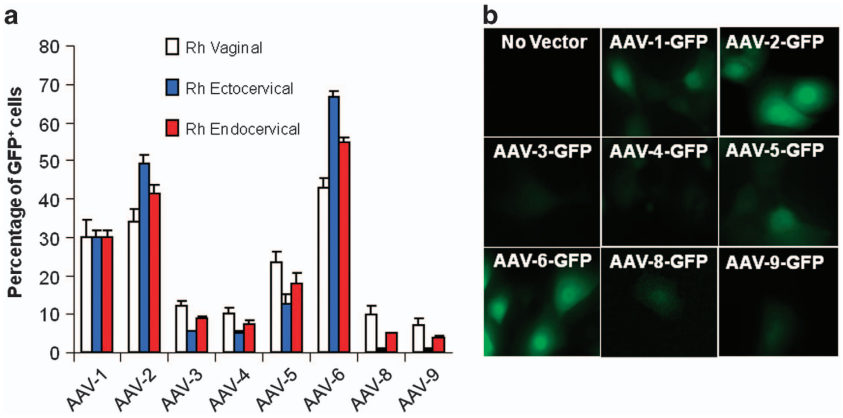

Figure 2. (**a** and **b**) Transduction of Rh macaque endocervical, ectocervical and vaginal epithelial cells by various AAV serotypes expressing GFP at MOI of 2×10^5 vg per cell. (**a**) Expression of GFP protein by transduced cells was detected by FACS, and presented as percentages of GFP+ cells. 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.

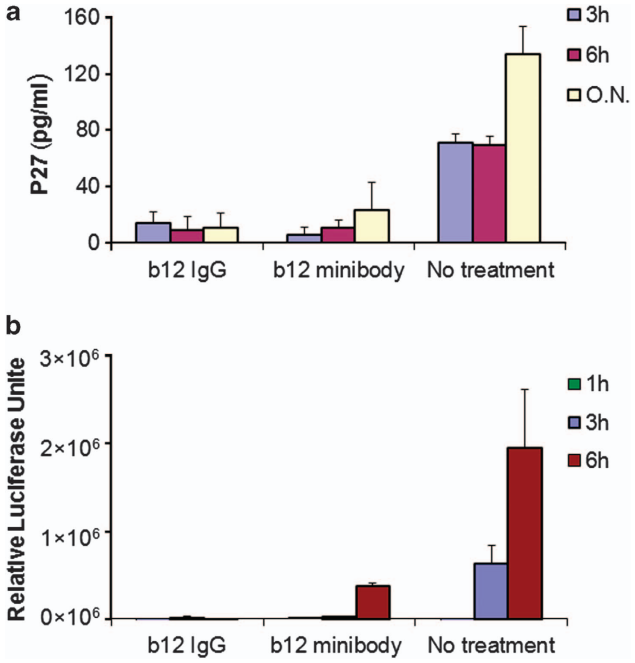

Figure 3. (**a** and **b**) Inhibition of SHIV162p4 virus transfer and infectivity by b12 minibodies across a monolayer of Rh PGECs (vaginal). After preincubating b12 minibodies or full-length b12 IgG (10 μg ml^{-1}) with SHIV162p4 (30 ng) for 1 h, medium from the basal chambers was collected at different 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.

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 per cell) 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.

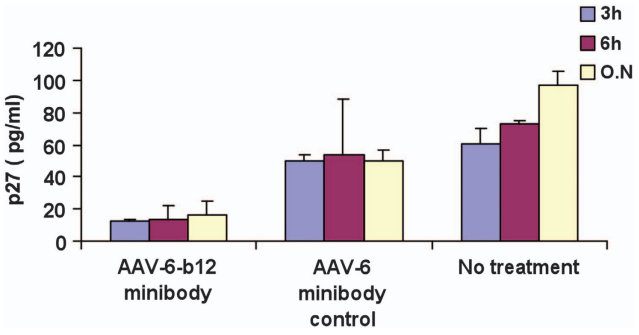

Figure 4. In *vitro* SHIV162p4 virus challenge. AAV-6-b12 minibody gene or irrelevant minibody gene control at 5×10^{10} particles were applied to the apical surface of Rh macaque genital epithelial monolayers for transduction. Four days later, SHIV162p4 (5 ng) was applied on the apical surface of the 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 statistically fewer SHIV162p4 virus particles compared with the untreated samples.

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 1×10^5 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 Pap smear cervical 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 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.

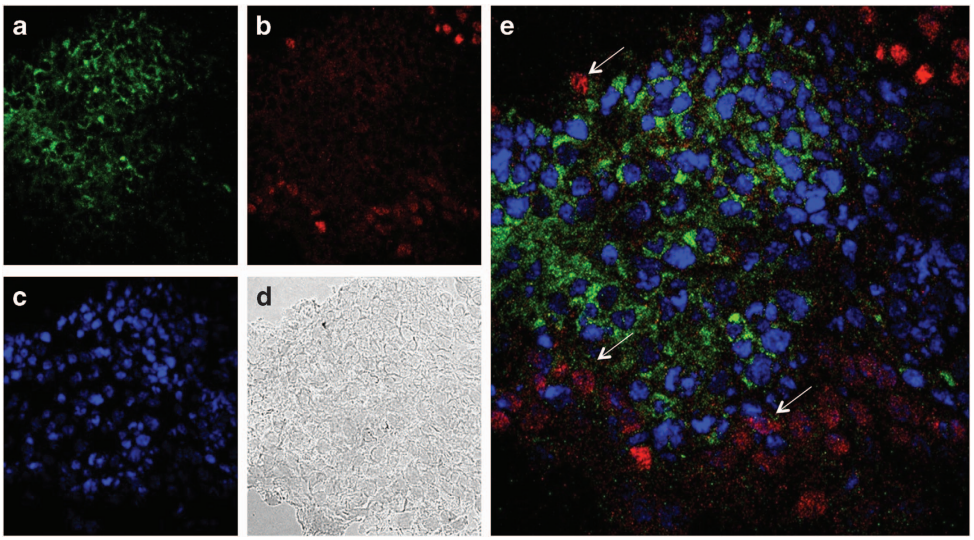

Figure 5. *In vivo* transduction of Rh macaque female genital tract with AAV-6-GFP. Fluorescence microscopy was used to examine cervical biopsy sections. A mucosal biopsy tissue sample with GFP-transduced cells (green, **a** and **e**) taken 1 week after transduction with AAV-6-GFP is shown; p63 immunofluorescence staining (red, **b** and **e**) highlights the basal epithelial cell layer. A transduced basal cell is indicated (arrow, **e**). Nuclei are stained with 4′,6-diamidino-2-phenylindole (blue, **c** and **e**). Differential interference contrast (**d**). Main images at x40, insets at x80.

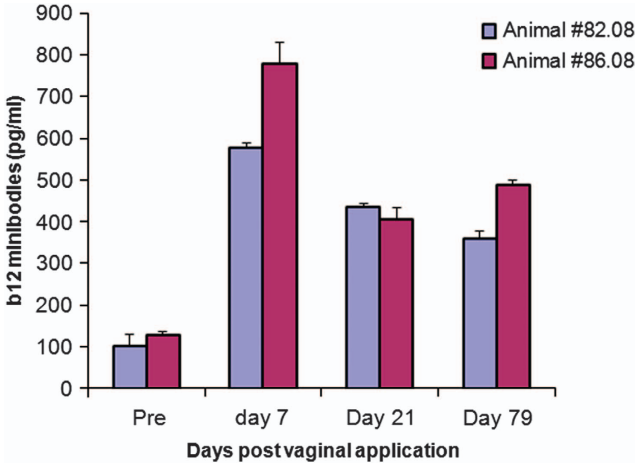

Figure 6. Concentration of b12 minibodies in vaginal secretion subsequent to *in vivo* transduction with AAV-6-b12. Vaginal secretions from each animal were absorbed to cellulose wicks. Samples were taken at different time points after AAV-6-b12 transduction. The concentration of b12 minibodies in vaginal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

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 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 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.[50] 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 miniendoscopic imaging.[51] 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.[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 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 0.5×10^{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[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 pericoital microbicide applications.

We anticipate application of higher doses such as 10^{13} or 10^{14} gc per 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 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.[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.[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 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.[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.[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 AAV-2/9 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] 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.[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 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.

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, USA), supplemented with bovine pituitary extract and recombinant human epidermal growth factor. The medium was further supplemented with 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and $CaC1_2$ 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).

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 CXCR4+ 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 (*tat* 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_2 humidified incubator. The R-tropic SHIV162p4 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] which was a gift from Dr Cheng-Mayer 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 IACCUC regulations. Tissues were collected in cold Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} and supplemented with penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}) and gentamicin

($50\ \mu g\ ml^{-1}$) (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 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) inT-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 passed two times before 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 Ca^{2+}-supplemented (0.4 mM $CaCl_2$) 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 once-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 speculum 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 (0.5×10^{12} 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 speculum-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, 5×10^4 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} gc and at MOI of 2×10^5 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.

Production of b12 minibodies and gp120 ELISA-binding assay

To produce the b12 minibodies, 293 T cells were transfected with pTR-b12scFvFc using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Sixteen hours after transfection, the media were 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, USA) according to the manufacturer's instructions. The b12 minibody proteins were eluted with IgG elution buffer (Thermo Scientific, Waltham, MA, USA) and the buffer was exchanged in PBS using Amicon Ultra-15 centrifugal filtration units (30 kDa molecular weight cutoff; Millipore, New Bedford, MA, USA). Concentration of the purified 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 10^5 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

SHIV162p4 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) 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, 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 $40\ \mu g\ ml^{-1}$, 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 (500μg ml − 1) of Sulfo-Tag-labeled goat anti-human IgG secondary antibody (Meso Scale Discovery, Gaithersburg, MD, USA; cat. no. 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 an MSD sector imager, Model no. 2400.

Immunofluorescence and immunochemistry

Immunofluorescence analysis was performed on Rh ectocervical, endocervical 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 (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 (poly IgA receptor) monoclonal antibodies. Cells were then examined under fluorescence microscope. *Immunochemistry*: 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-methylbutane (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 SP5 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.

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