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Monoclonal And Single Domain Antibodies Targeting β-Integrin Subunits Block Sexual Transmission of HIV-1 in *in vitro* and *in vivo* Model Systems

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Abstract

Background—Poor adherence to prevention regimens for gel-based anti-HIV-1 microbicides has been a major obstacle to more effective pre-exposure prophylaxis. Concern persists that the antiretroviral drug containing microbicides might promote development of antiretroviral resistance.

Methods—Using in vitro transwell systems and a humanized mouse model of HIV-1 sexual transmission, we examined, as candidate microbicides, antibodies targeting the heterodimeric leukocyte function associated antigen 1 (LFA-1), a non-virally encoded protein acquired by the virus that also plays a critical role cell movement across endothelial and epithelial barriers. LFA-1 specific single domain variable regions from alpaca heavy-chain only antibodies (VHH) were identified and evaluated for their ability to inhibit HIV-1 transmission in the *in vitro* transwell system.

Results—Monoclonal antibodies targeting the CD11a and CD18 components of LFA-1 significantly reduced cell-free and cell-associated HIV-1 transmission in the *in vitro* transwell culture system and prevented virus transmission in the humanized mouse model of vaginal transmission. The broadly neutralizing monoclonal antibody b12 was unable to block transmission of cell-free virus. CD11a-specific VHH were isolated and expressed and the purified variable region protein domains reduced *in vitro* transepithelial transmission with an efficacy comparable to that of the CD11a monoclonal antibody.

Conclusions—Targeting integrins acquired by HIV-1 during budding and which are critical to interactions between epithelial cells and lymphocytes can reduce viral movement across epithelial barriers and prevent transmission in a humanized mouse model of sexual transmission. VHH capable of being produced by transformed bacteria can significantly reduce transepithelial virus transmission in *in vitro* model systems.

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Conflicts of Interest: The authors declare no conflicts of interest.

Introduction

HIV-1 prevention studies analyzing the prophylactic use of antiretroviral agents have demonstrated varying levels of efficacy, with adherence to prophylactic regimens being a major source of failure[1–3]. Additionally, whether antiretroviral-based microbicides are effective against vaginal transmission of cell-associated HIV, which is readily found in seminal and vaginal fluids[4–6], is unknown.

The role of the β-integrin leukocyte function-associated antigen-1 (LFA-1) and its counter-receptor Intercellular Adhesion Molecule-1 (ICAM-1) in movement of cells across endothelial and epithelial barriers is well-described[7–16]. ICAM-1 is expressed on both cervical and vaginal epithelium[17], potentially facilitating transmigration of HIV-1 infected lymphocytes and monocytes.

LFA-1 is a heterodimer consisting of an alpha chain, CD11a, and a beta chain, CD18. CD11a contains the conserved 200 amino acid I-domain, which is responsible for ligand binding[18,19]. Both ICAM-1 and LFA-1 have been demonstrated to be acquired by the HIV-1 virion as it buds from infected cells[20–23]. In the current study we have examined the potential efficacy of targeting this interaction to interrupt sexual HIV-1 transmission.

Materials and Methods

Cell lines and antibodies

HT-3 cervical epithelial cells were obtained from the American Type Tissue Collection (ATCC, Manassas, VA). Jβ2.7 LFA-1+ and LFA-1- Jurkat cells were kindly provided by Catarina Hioe (NYU, New York, NY). Anti-CD18 monoclonal antibody (Mab), H52, was a gift from Dr. James Hildreth (University of California, Davis, Davis, CA). Anti-CD11a Mab (38) was purchased from Abcam (Cambridge, MA). The broadly neutralizing anti-gp120 Mab, b12,[24] was kindly provided by Dr. Dennis Burton (The Scripps Institute, La Jolla, CA). IgG Isotype control was purchased from Becton Dickinson (Franklin, Lakes, NJ). FITC conjugated goat anti-mouse IgG was purchased from Jackson ImmunoResearch Laboratories (Westgrove, PA). Anti-T7 tail fiber Mab was purchased from Novagen (San Diego, CA). Anti-His Mab was purchased from GE Healthcare Biosciences (Pittsburgh, PA). HRP-conjugated goat anti-mouse IgG was purchased from Sigma-Aldrich (St. Louis, MO).

Flow Cytometry

Jβ2.7 cells were stained with anti-CD11a (1:1000) in 3% BSA (Sigma-Aldrich, St. Louis, MO) in PBS at 4°C for 1 hr. Cells were washed twice with cold PBS and FITC conjugated goat anti-mouse IgG was added to cells at 4°C for 30 min. Cells were washed twice, resuspended in 1% paraformaldehyde and analyzed using the Becton Dickinson FACSCalibur and the Cellquest program (BD Biosciences, San Jose, CA). Data were analyzed using Flojo software (Ashland, OR).

Human cervical epithelial transwell cultures

HIV-1 infected human PBMC were prepared as previously described[25]. For epithelial cell transwell cultures, 600 μl cMcCoy's media was added to wells in a 24 well tissue culture plate (Becton Dickinson, Franklin Lakes, NJ). HT-3 cells were plated at 5×10^5 cells/well in 12-mm diameter transwell inserts with a pore size of 3μm (Millipore, Billerica, MA) and placed into each well. Transwells were maintained at 37°C, 5% CO2. Media was replaced with cRPMI every two days. Confluency of the cervical epithelial monolayer was confirmed by monitoring the permeability to horse radish peroxidase (HRP, Sigma-Aldrich, St. Louis, MO); cells were considered confluent when ≤ 1% of the HRP could be recovered from the basal compartment.

Cell-associated HIV-1 in vitro transmigration studies

Transmigration studies were performed as previously described[25].

Cell-free HIV-1 in vitro transmigration studies

1×10^6 PHA stimulated huPBMC in cRPMI with 10 u/mL IL-2 were infected with 10^5 $TCID_{50}$ $HIV\text{-}1_{BaL}$, originally grown in primary human monocyte/macrophage cell culture (Advanced Biotechnologies, Columbia, MD). After one week HIV-1 (2.6 ng p24) from these cultures was mixed with the indicated antibodies for 1 hr at 37°C and then placed into the apical chamber of transwells.

Jβ2.7 LFA-1+ and LFA-1- control cells were transfected with pNL4-3 by electroporation. Two ng p24 LFA-1+ or LFA-1- HIV-1 obtained from these cell cultures was added to the apical side of cervical epithelial monolayers and incubated at 37°C. For both sets of experiments p24 levels in the lower chamber were determined after 24 hr.

Cell-associated HIV-1 transmission in HuPBL-SCID mouse models

The HuPBL-SCID mouse model, with known patterns of human cell distribution[26], was used as previously described[25]. Reconstituted and progestin-treated mice were intravaginally administered 10 μl of the indicated antibodies prepared in PBS and 1% BSA (Sigma-Aldrich, St. Louis, MO) 5 min before vaginal administration of 1×10^6 $HIV\text{-}1_{BaL}$ infected HuPBMCs. The five minute pre-challenge interval for antibody administration was selected to mimic a setting in which constitutively-produced VHH would be present. Two weeks later, HuPBMCs were recovered from the peritoneal cavity of euthanized mice by cold PBS lavage.

Recovered cells were cultured with 1×10^6 PHA-stimulated normal PBMCs in cRPMI and 10 U/mL IL-2 for one week. HIV-1 positivity was determined by p24 ELISA of supernatant and p17 gag PCR amplification from cultured cells[27].

CD11a I-domain production

CD11a I-domain/pET20 was kindly provided by Timothy Springer (Harvard Medical School, Boston, MA). The sequence contained two mutations (F265S/F292G) that conferred increased affinity to ICAM-1. The DNA construct was transformed into BL21 DE3 cells (New England Biolabs, Ipswich, MA) and colonies grown on LB agar overnight at 37°C.

Selected colonies were grown in 800 ml LB media to an OD of 0.8 before being induced with 1 mM IPTG for 4 hr at 37°C. Cultures were centrifuged and pellets were stored in −20°C. CD11a I-domain purification was previously described[19].

Alpaca PBMC isolation and cDNA preparation

Alpacas from a local breeder were housed at a large animal facility maintained by Johns Hopkins University. PBMC obtained from Ficoll Hypaque centrifugation of peripheral blood was stored in 10^6 cells/mL aliquots in RNAlater (Ambion, Norwalk, CT) at −80°C. Thawed cells were resuspended in 1 mL of TRIzol Reagent (invitrogen). RNA was extracted in chloroform, precipitated with isopropanol, washed in ethanol, dissolved in DEPC treated water (Quality Biological, Gaithersburg, MD) and stored at −80°C. First-strand cDNA synthesis was performed using SuperScriptIII first strand cDNA synthesis reverse transcription kit (Invitrogen, Grand Island, NY) according to the manufacturer's protocol. cDNA was stored at −20°C.

VHH cloning and T7 VHH library construction

VHH isolation was performed as previously described[28]. A T7 VHH library was produced using a cloning and packaging kit provided by Novagen (San Diego, CA) following the manufacturer's protocol. Briefly, the *vhh* was ligated into T7Select10-3b arms and packaged into T7 phage. The resulting library was amplified in BLT5403 *E. coli* for the inserted *vhh* gene to be expressed on the viral capsid.

Biopanning and VHH selection

CD11a I-domain (0.5 μg/ml) was immobilized overnight at 4°C in a 96 well immunoplate (NUNC, Thermo Fisher Scientific, Waltham, MA) and blocked with 5% milk in TBS for 2 hours at RT. 10^{10} phage from the phage library was added to each well overnight at 4° C. Wells were washed 12X with 1X TBS with .1% Tween-20 (TBS-T) followed by 12 washes with TBS. Bound phage were eluted with 1% SDS in TBS and added to BLT5403 *E.coli* for further amplification. Amplified phage were used for another round of biopanning. Phage eluted in the final step were added to BLT5403 *E.coli* and plated onto LB agar plates for plaque purification.

Individual phage were screened against CD11a I-domain for further selection using a phage ELISA. Briefly, CD11a I-domain was immobilized overnight at 4° in a 96 well immunoplate (NUNC, Thermo Fisher Scientific, Waltham, MA). The following steps were performed at room temperature. Wells were blocked with 2% BSA in PBS for 1 hour. Phage were added directly to each well for 2 hr. Wells were washed 5X with 1X PBS and .05% Tween-20 (PBS-T) between each step in the assay. Bound phage were detected by the addition of anti-T7 tail fiber Mab for 1 hr. followed by the addition of HRP conjugated anti-mouse IgG Mab for 1 hr. ABTS Peroxidase Substrate System (KPL, Gaithersburg, MD) was added for up to 30 minutes and plates were read at 405 nm in a Synergy HT plate reader (Biotek, Winooski, VT). VHH binding to CD11a was screened using the same protocol except that 0.5 μg of the indicated antibody or VHH was added to the microwell instead of phage.

Soluble VHH production

VHH sequences were subcloned into the pET47b plasmid (Novagen, San Diego, CA) using primers included in the T7 cloning kit provided by Novagen. Vhh/pET47b plasmids were transformed into *E. coli* BL21 DE3 (New England Biolabs, Ipswich, MA). Cultures were grown overnight at 37°C, diluted into 800 mL of LB Broth (LB), grown to an OD of 0.8, induced with IPTG at a concentration of 1mM and grown for an additional 4 hr. Culture pellets were stored at −20°C. Pellets were thawed on ice and resuspended in 10 ml lysis buffer (100 mM NaH_2PO_4, 10mM Tris Base, 6M GuHCl, 10mM Imidazole, pH 8.0) and placed in −80° for 30 min to lyse cells. The frozen lysis mixture was thawed at room temperature, 30 ml lysis buffer was added and lysis continued at room temperature for 2 hr. Mixtures were centrifuged at 14000 rpm for 30 min at 4°C. Supernatants were added to 1 mL Ni-NTA agarose (Qiagen, Germantown, MD) and rocked for 1 hr at room temperature. The mixture was added to Poly-Prep chromatography columns (Bio-Rad, Hercules, CA). Agarose was washed with 2 column volumes of denaturing buffer (100 mM NaH_2PO_4, 150 mM NaCl, 8 M Urea, 20 mM Imidazole, pH 8.0) followed by 7 column volumes of renaturing buffer (50 mM NaH_2PO_4, 500 mM NaCl, 20 mM Imidazole). His-VHH was eluted with 3 mL elution buffer (50 mM NaH_2PO_4, 500 mM NaCl, 250 mM Imidazole, pH 8.0). The eluted protein was dialyzed overnight in 1X PBS.

Soluble VHH ELISA

CD11a I-domain (0.5 μg/ml) was immobilized overnight at 4°C in a 96 well immunoplate (NUNC, Thermo Fisher Scientific, Waltham, MA). The following steps were performed at room temperature, with 5× washes occurring between each incubation step. Wells were blocked with 2% BSA in PBS for 1 hr. His-VHH was added to each well for 1 hr, followed by the addition of anti-His Mab for 1 hr. HRP conjugated anti-mouse was added for 1 hr. followed by addition of the ABTS Peroxidase Substrate (KPL, Gaithersburg, MD) for up to 30 minutes. Plates were read at 405nm in a Synergy HT plate reader (Biotek, Winooski, VT).

Statistics

A one-way analysis of variance with Bonferroni correction was used to determine the significance of differences in p24 concentrations. A Fisher exact test was used to evaluate the significance of differences in infection rates among challenged mice. Correlation data were determined using pwcorr. All statistical analyses employed Stata software (College Station, TX).

Results

Antibodies against CD11a and CD18 prevent the transmigration of cell-associated HIV-1 across a cervical epithelial monolayer

Anti-CD11a (38), anti-CD18 (H52), or isotype control mouse IgG1 were assayed as described for their ability to block migration of infected PBMC. At concentrations of 0.02 μM and .004 μM, both anti-CD11a, 38, which specifically binds to the I-domain and blocks ligand binding, and anti-CD18, H52, which binds to the hybrid region of CD18 and prevents

LFA-1 activation[29,30], significantly reduced both transmigration of PBMCs across the cervical epithelial transwell and HIV-1 p24 levels in the lower chamber of the transwell compared to the IgG controls ($p<0.001$) (Fig. 1A, 1B). There was a significant correlation ($r=0.72$, $p=0.001$) between reduction in cell levels and p24.

Targeting LFA-1 reduces cell-free HIV-1 transmission

Host-derived LFA-1 is reportedly incorporated into the viral membrane during budding[20–23]. To evaluate the role of host-derived LFA-1 on migration of cell-free virus across epithelial surfaces, we produced HIV-1 from Jβ2.7 cells lacking LFA-1 (Fig. 2A), and from Jβ2.7 cells constitutively expressing LFA-1 (Fig. 2B). Virus from these two sources in equivalent concentrations (2 ng in 1 ml) was added to the apical side of cervical epithelial transwells and viral transmission to the basilar compartment was assayed after 24 hr. Overall transmission of free virus across the epithelial barrier was inefficient with less than 3% of the LFA+ HIV-1 in the apical compartment reaching the basilar compartment. Nevertheless, compared to LFA-1+ HIV-1, LFA-1-HIV-1 had a significant reduction (35%) in p24 concentrations attained in the basilar compartment ($p=0.0009$) (Fig. 2C).

In a related experiment, we then examined the comparative ability of anti-CD-11a antibody or the broadly neutralizing gp120-specific b12 Mab[24] at 0.1 μM concentration to block transmission of PBMC derived HIV-1 (Fig. 2D). Virus (2.6 ng p24) was incubated with 1 ml. of the indicated antibodies for one hour prior to the application of the mixture to the apical side of cervical epithelial transwells. Again free virus transmission across the epithelial barrier was inefficient with less than 5% of the added virus reaching the basilar compartment in the group treated with IgG. However, after 24h p24 concentrations in the lower chambers were significantly reduced (54%) in the anti-CD11a treated group compared to control IgG1 treated groups ($p < 0.01$). Interestingly, the broadly neutralizing b12 antibody did not reduce cell free virus transmission across the epithelial barrier when compared to IgG.

In vivo protective efficacy of anti-CD11a and anti-CD18 in the HuPBL-SCID Mouse Model

We next evaluated the ability of anti-CD11a and anti-CD18 Mab to protect Hu-PBL- SCID mice from vaginal HIV-1 transmission. Mice received 10 μl of either 5 or 20 μg/ml of the antibodies intravaginally five minutes prior to challenge with 10^6 HIV-1 infected PBMCs obtained from a source that differed from the originally transplanted PBMC (Table 1). Cell-associated transmission was evaluated because cell-free virus cannot be transmitted in this model system. Two weeks following challenge, the mice were euthanized, and human PBMC were recovered from the peritoneal cavity and assayed for HIV-1 infection as described in the Methods. In separate experiments, both anti-CD11a (5 μg/ml) and anti-CD18 (20 μg/ml) completely protected against HIV-1 infection (Table 1, $p<0.01$ compared to IgG controls).

Selection of VHH against the CD11a I-domain from a T7 phage library

An alternative method for delivering antibodies would be their constitutive production and secretion by the transformed bacterial flora of the vagina, which consists primarily of

bacteria of the genus *Lactobacillus*. Studies from other laboratories have shown that lactobacilli can constitutively express and secrete VHH[31–35].

To address the feasibility of developing biologically active VHH against the CD11a I-domain, we screened an alpaca PBMC-derived VHH T7 phage display library. We selected for VHH against the CD11a I-domain rather than CD18 due to the availability of soluble recombinant and conformationally correct CD11a I-domain[19,36]. We performed two rounds of phage biopanning against recombinant CD11a I-domain. Eighty individual phage plaques recovered from the biopanning process were evaluated for binding in a phage ELISA. Of the 80, five had binding to the CD11a I-domain that was significantly above that of the control (data not shown). Sequencing of the complementarity-determining regions of the 5 VHH indicated they were all unique (Table 3). The VHH from these five phage were cloned into an *E. coli* BL21 bacterial expression vector for soluble VHH expression and purification. Only two of the purified VHH, VHH55 and VHH60, differed significantly from the IgG control ($p<0.001$, Figure 3A) by CD11a I-domain ELISA. In this analysis VHH60 had significantly greater binding than VHH55 ($p=0.01$, Figure 3A), but those two VHH and the anti-CD11a antibody all differed significantly from the IgG control ($p<0.001$).

We then evaluated the efficacy of VHH55 and VHH60 to prevent transmigration of HIV-1-infected PBMCs in the *in vitro* transwell assay. The indicated VHH, anti-CD11a, or a mouse IgG1 isotype control were mixed with HIV-1-infected PBMCs 1h prior to their addition to the apical side of cervical epithelial transwells. As seen in Figure 3B, after 24 hours VHH 55, VHH 60 and the anti-CD11a Mab at concentrations of 4 μg/ml significantly reduced transmitted HIV-1 p24 compared to the IgG control ($p<0.001$). At the lower concentrations of 0.4 μg/ml, only VHH 60 and the anti-CD11a Mab achieved significant reduction of transmitted p24 compared to the IgG control ($p<0.001$). At this concentration VHH 55 was less effective and did not significantly differ from the IgG negative control ($p=0.377$).

Discussion

In these studies we have demonstrated that antibodies to the heterodimeric components of LFA-1 are capable of reducing both cell-associated and cell-free HIV-1 transmission in an *in vitro* transwell system. The observed *in vitro* reduction in cell- associated transepithelial transmission of HIV-1 of 51% (0.4 μg/ml) and 72% (4 μg/ml), correlated with nearly complete interruption of HIV-1 transmission in an *in vivo* model of vaginal transmission in Hu-PBL-SCID mice. The current studies indicate a specific role for LFA-1 in initial events leading to sexual transmission of cell-associated virus in an *in vivo* model system.

Our studies in mice were facilitated by the ability of human LFA-1 to bind murine ICAM-1; however, the converse is not true[37]. In addition, anti-human CD11a and anti- human CD18 do not cross-react with mouse LFA-1; therefore the *in vivo* protection we observed reflects the role of human LFA-1 on the transmission of HIV-1 infected human cells. Previous studies from our group have demonstrated that human PBMC inoculated vaginally can be detected in periaortic lymph nodes of Hu-PBL-SCID mice within four hours of inoculation. However, human PBMCs injected peritoneally migrate and engraft into peritoneal mesentery with few cells migrating to the spleen, and none populating the vaginal tract[26].

We have also studied the effect of antibodies against LFA-1 on cell-free viral transmission. As it buds from host cells, HIV-1 acquires surface expressed adhesion molecules and integrins, including LFA-1[38], which has been shown to increase HIV-1 infection of T-cells[22]. The mechanism by which cell-free HIV-1 might cross the layer(s) of epithelial cells lining the vagina and cervix is not established and several relevant mechanisms might contribute to this process, including disruption of cell junctions by HIV-1 envelope protein[39], engagement of ICAM-1 signaling pathways by LFA-1[11], or by transcytosis through epithelium, although the relevance of this latter model is controversial[40–42]. However, the finding that HIV-1 produced from LFA-1 deficient cells had reduced transmigration compared to HIV-1 produced from cells expressing LFA-1 suggests that interactions between LFA-1 and cell adhesion molecules on cervical epithelial cells can play a role in HIV-1 transmission.

That b12 did not reduce recovery of virus from the basilar chamber of transwells indicates that viral proteins involved in neutralization are distinct from those that play a role in viral movement across an epithelial cell barrier. Cell-free virus transmission could not be evaluated in our *in vivo* Hu-PBL SCID model system because, in the absence of susceptible human cells in the perivaginal region, transvaginal infection cannot be established in this model system.

It has previously been shown that antibodies against CD11a of LFA-1 prevent the transmigration of monocyte associated HIV-1 under pro-inflammatory conditions and that LFA-1 was interacting in these studies with ICAM-2 and ICAM-3, but not ICAM-1[43]. However, lymphocytes, which were the predominant cell in our transmission studies (data not shown), account for the majority of cell-associated HIV-1 found in seminal fluid[4]. In the non-inflammatory setting of our studies, current and previous findings[25] clearly implicate LFA-1 and ICAM-1 in the cellular transmission process.

CD11a is also expressed on dendritic cells that play a role in conception, in transmission of sexually transmitted infections and in the initiation of adaptive immune responses within the genitourinary tract[44]. While activity against dendritic cells could theoretically interfere with the development of adaptive immune responses, adaptive responses have already evolved to be attenuated within the GU tract {Clark, 2013 #790}, presumably to reduce the risk of rejection of the fetus and sperm.

One mechanism by which antibodies might be introduced into the female genitourinary tract to prevent HIV-1 transmission would be their constitutive secretion by the commensal bacterial flora of that site, primarily different species of *Lactobacilli*. The pH stability of VHH might prove particularly important in this setting, as will their ease of expression in conformationally correct form by bacteria, especially as compared to the commonly employed single chain variable region antibody constructs (scFv)[45]. Constitutive expression of VHH at sufficient concentrations would obviate any concerns about the half-life of these molecules within the genitourinary tract. Bacterial expression and secretion of VHH has been demonstrated to be protective against pathogen challenge in multiple model systems[31,33–35,45]. *Lactobacilli* cannot sustain colonization within the murine genitourinary tract during diestrus, the phase most favorable to HIV-1 infection in our mouse model[46], but

in the human setting, in which colonization with lactobacilli uniformly occurs in healthy women, the potential efficacy of VHH could be evaluated. Our *in vitro* studies indicated that VHH 60 achieved inhibition of cell-associated HIV-1 transmission that was equivalent to that observed with the anti-CD11a Mab that was effective *in vivo*.

Previous studies have shown that VHH are not immunogenic and do not disrupt the integrity of the epithelial layer[47,48], reducing their likely exposure to the underlying lymphoid tissue. Colonization by transformed lactobacilli might permit stable, constitutive expression of VHH targeting CD11a, reducing the need for frequent applications, while providing protection that would be transparent to all users and effective against both cell-associated and cell-free virus.

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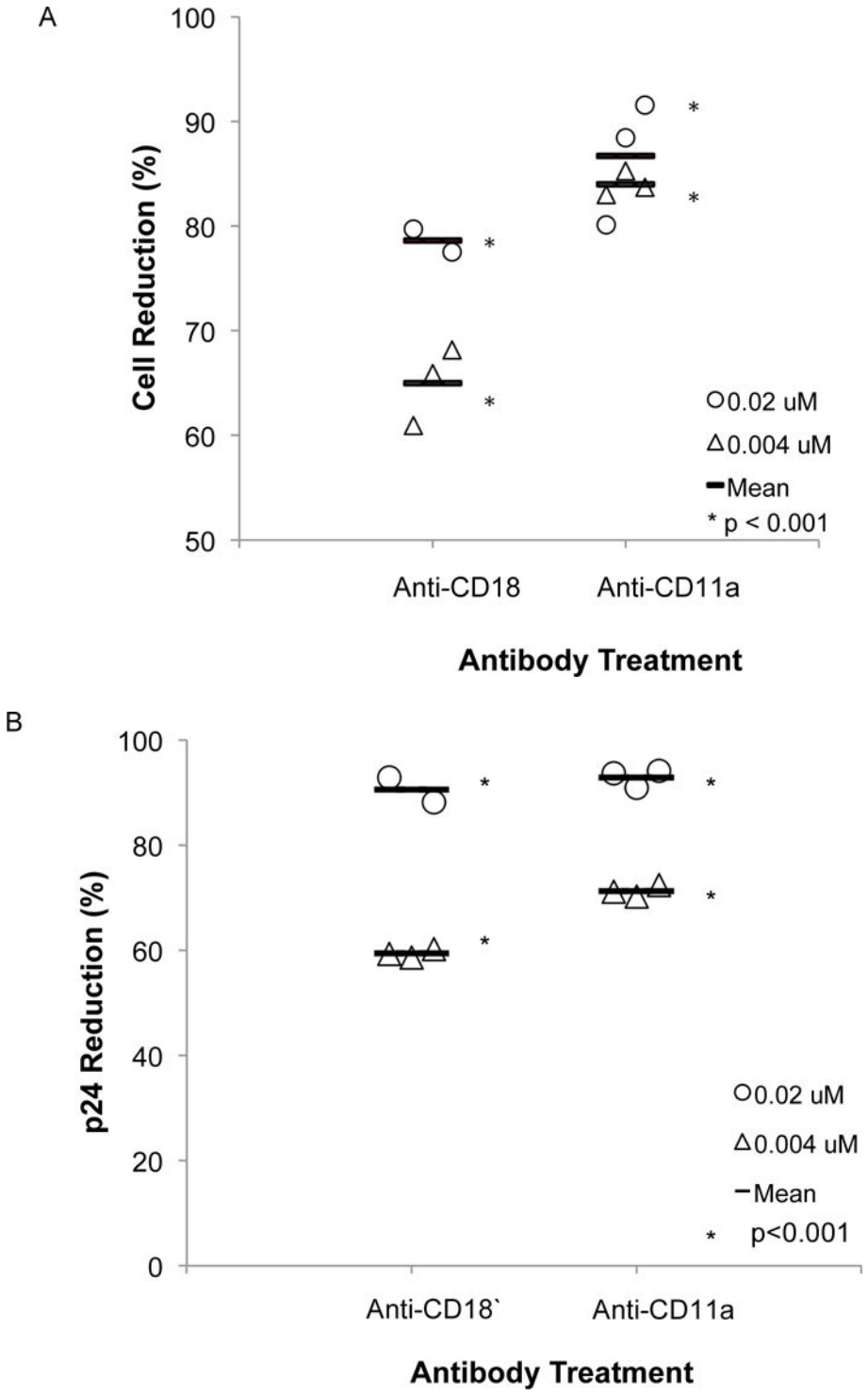
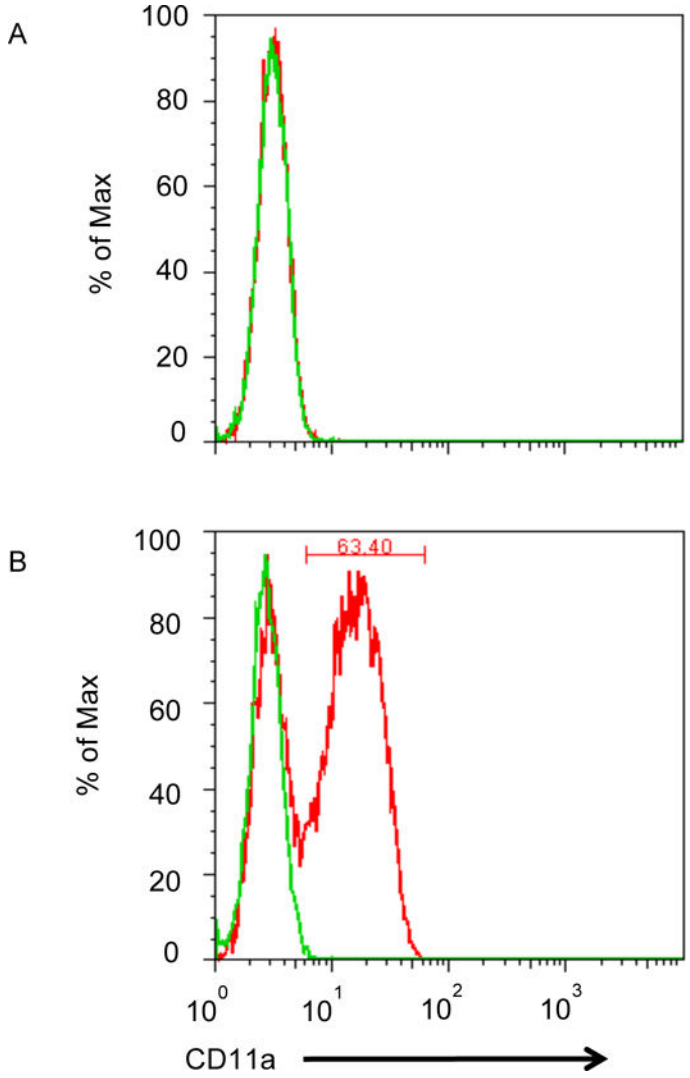


Figure 1. Effect of Mab against LFA-1 on transmission of cell-associated HIV-1 across an epithelial monolayer

Mouse anti-human CD11a (38), mouse anti-human CD18 (H52), or mouse IgG1 at concentrations of 0.02 μM or 0.004 μM were mixed with 5×10^5 HIV-1-infected PBMCs 1 hr prior to the addition of the mixture to the apical side of HT-3 cervical monolayers grown

on permeable transwell supports. After 24h (A) cells in the basal compartment were counted and (B) p24 concentrations in the basal compartment were measured and percent reduction was determined compared to control IgG. All groups treated with a Mab had significantly lower number of cells and of p24 concentrations in the basal chamber compared to the IgG control group. The reduction in cell number significantly correlated (r=0.73, p=0.001) with the reduction in p24 concentration.



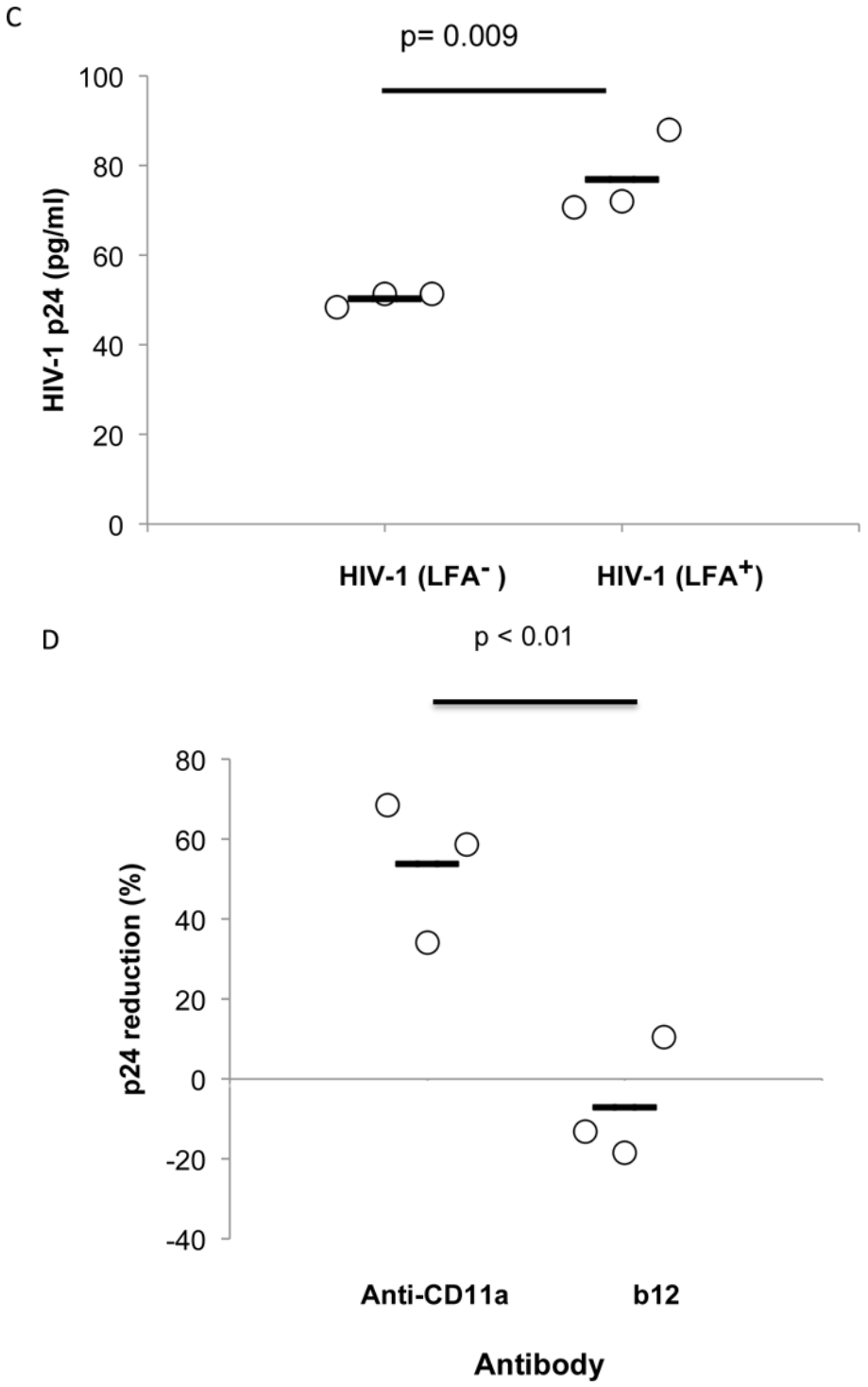


Figure 2. HIV-1 From LFA-1 Expressing Cells Crosses Epithelium with Greater Efficiency than HIV-1 from Cells Lacking LFA-1 Expression

Jβ2.7 LFA-1- and Jβ2.7 LFA-1+ cells were used to produce HIV-1. The expression or absence of CD11a on the cells was tested by flow cytometry. A) Jb2.7 LFA-1- did not express CD11a. B) 63.40% of Jβ2.7 LFA-1+ cells expressed CD11a. For producing HIV-1, plasmid NL4-3 was transfected into these cells by electroporation. The production of HIV-1

was detected by viral p24. Based on p24 determinations, a 100 μl solution containing 2000 pg of HIV-1 was added to the apical compartment of the transwell. C) After 24 hours, HIV-1 transmisson was evaluated by detecting viral p24 in the basal chamber. The virus produced from Jβ2.7 (LFA-1+) showed significantly greater transepithelial transmission compared to that from Jβ2.7 (LFA-1-), p=0.009. D) HIV virions produced from PBMC infection were mixed with mouse anti-human CD11a (38), anti-gp120 (b12), or mouse IgG1 at a concentration of 0.1 μM 1 hour prior to their addition to the apical side of transwell inserts. After 24 hours basal p24 levels were measured and percent reduction compared to IgG control were calculated. Reduction in p24 levels compared to the IgG1 control was observed only in anti-CD11a treated groups ($p < 0.01$).

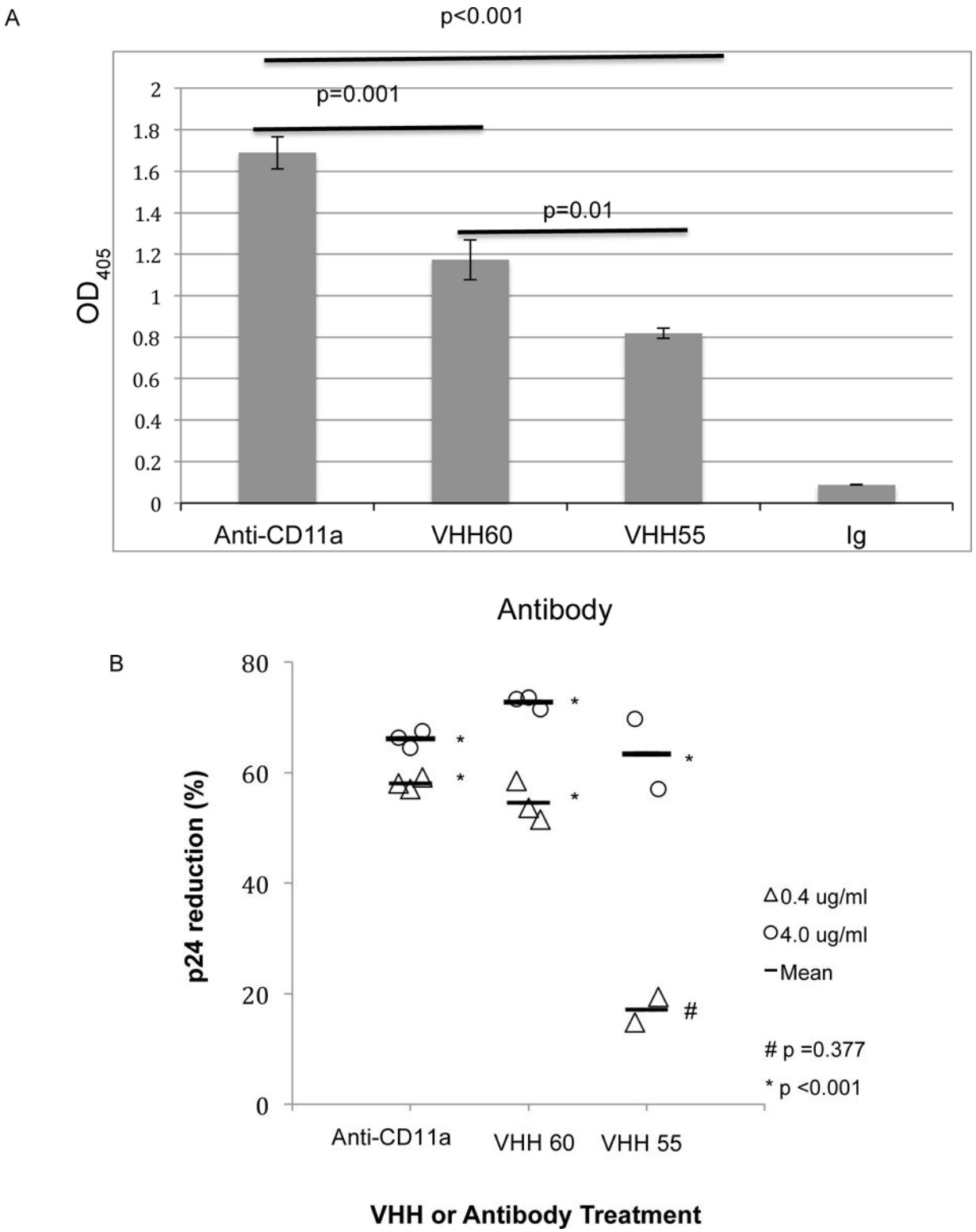


Figure 3. VHH against the CD11a I-domain reduces *in vitro* cell-associated HIV-1 transmission

(A) ELISA measurement of binding of 0.5 μg of different VHH or anti- CD11a Mab. The relative binding affinity, as measured by OD_{405}, indicated that VHH55 bound with

significantly less affinity than either anti-CD11a or VHH60, although all bound with significantly greater affinity than the IgG control ($p<0.001$). Not shown in the figure were three additional VHH included in the experiment, all of which had OD_{405} levels below 0.26, which did not differ significantly from the IgG control ($p=1.000$). (B) VHH 55 and VHH 60, normal IgG or an anti-CD11a Mab (38) were mixed with 5×10^5 HIV-1 infected, PHA activated PBMCs 1 hr prior to application to the apical side of HT-3 cervical epithelial transwell inserts. After transmigration was allowed to occur for 24 hr, basal side p24 concentrations were measured and the percent reduction compared to control IgG was determined. This experiment is representative of three experiments in which the reduction in HIV-1 transmission to the basal compartment resulting from co-incubation with VHH 60 at 0.4 or 4.0 μg/ml differed significantly from the IgG control. Compared to the the IgG control VHH 60 at a concentration of 0.4 μg/ml reduced p24 concentrations in the three separate experiments by 74%, 58% (this experiment) and 51%. VHH 55 significantly reduced p24 concentrations at a concentration of 4.0 μg/ml but significant reduction was lost at 0.4 μg/ml concentration.

Table 1

Ability Of Monoclonal Antibodies Targeting Either the CD11a or CD18 Components of LFA-1 To Block Cell-Associated HIV-1 Transmission In A Hu-PBL SCID Mouse Model

Treatment	Concentration (μg/mL)	HIV-1 positive Mice/Total Challenged	P-value
Mouse IgG1 Isotype			
Control	20	7/10	–
Human Anti-CD11a	20	1/10	p = 0.02
Human Anti-CD11a	5	0/10	p < 0.01
Mouse IgG1 Isotype			
Control	20	6/7	–
Human Anti-CD18	20	0/8	P < 0.01

Table 2

CDR sequences of the five VHH with highest anti-CD11a reactivity by ELISA

	CDR1	CDR2	CDR3
VHH 52	KSTLDEYS	ISASGFSI	AADRWL-------VCRGRQTTDFNT
VHH 55	GSALTYYT	LSSFQGRT	
	AAQASWTADSVQTMCDEMAPREYDI VHH 56 GNDFSIHN ISS-		
GGTT	KAEIVT------TPPPWYRETQFDV		
VHH 58	GSLSSINV	ISSGTST	NLDIT--------TTTMWLSQAY--
VHH 60	GFSLENKP	ISSTGDET	AVYLGG------GNCLSSLGHDY--