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A directed evolution approach to select for novel *Adeno-associated virus* capsids on an HIV-1 producer T cell line

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Abstract

A directed evolution approach was used to select for *Adeno-associated virus* (AAV) capsids that would exhibit more tropism toward an HIV-1 producer T cell line with the long-term goal of developing improved gene transfer vectors. A library of AAV variants was used to infect H9 T cells previously infected or uninfected by HIV-1 followed by AAV amplification with wild-type adenovirus. Six rounds of biological selection were performed, including negative selection and diversification after round three. The H9 T cells were successfully infected with all three wild-type viruses (AAV, adenovirus, and HIV-1). Four AAV *cap* mutants best representing the small number of variants emerging after six rounds of selection were chosen for further study. These mutant capsids were used to package an AAV vector and subsequently used to infect H9 cells that were previously infected or uninfected by HIV-1. A quantitative polymerase chain reaction assay was performed to measure cell-associated AAV genomes. Two of the four *cap* mutants showed a significant increase in the amount of cell-associated genomes as compared to wild-type AAV2. This study shows that directed evolution can be performed successfully to select for mutants with improved tropism for a T cell line in the presence of HIV-1.

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Conflicts of Interest

KJDAE is a member of the scientific advisory board, and DVS is a co-founder, the acting Chief Scientific Officer, and co-chairman of 4D Molecular Therapeutics, Emeryville, CA. DVS and JRW have patents related to AAV engineering.

Disclaimer

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Keywords

directed evolution; AAV; gene transfer; T cell; HIV-1

1. Introduction

Adeno-associated virus (AAV) is the third most popular gene transfer vector used today ranking just after adenovirus and retrovirus (Edelstein, 2017). Despite some recent controversy regarding its role in cancer (Nault et al., 2015; Park et al., 2016; Srivastava and Carter, 2017), AAV is generally considered to be a nonpathogenic virus. As of April 2017, AAV has been used in 183 clinical trials (Edelstein, 2017) with no reported cancers. AAV exhibits broad tropism, persistence, high transduction capability, lack of superinfection immunity, and high stability. It may be grown and purified to high titers, and it has the ability to infect dividing or nondividing cells (Daya and Berns, 2008). These characteristics make AAV an appealing choice for gene transfer applications. Recombinant AAVs are being investigated as vectors for clinical gene transfer to a wide variety of cells and tissues (Hasbrouck and High, 2008; Kota et al., 2009; Maguire et al., 2009; Maguire et al., 2010; Muzyczka, 1992; Srivastava, 2008; Wagner et al., 1999). Transduction with AAV vectors has been shown to result in long-term transgene expression *in vivo* in several cell types including skeletal muscle, photoreceptors, liver, and neuronal cells (Daya and Berns, 2008; Hasbrouck and High, 2008; Liu et al., 2007). As a result, AAV was used in the first regulatory approval of a gene therapy product in Western nations, in 2012 within the European Union.

AAV is a member of the *Parvoviridae* family. As a member of the *Dependoparvovirus* genus, this virus requires the help of another virus in order to replicate. Viruses known to help AAV include adenovirus, herpesvirus, and poxvirus. In the absence of a helper virus, AAV cannot fully replicate, but it can infect cells and deliver a foreign gene of interest (a transgene), which is the desired function of a viral vector. The wild-type virus is a 4.7 kb single-stranded DNA virus that contains two genes: *rep*, which encodes four proteins required for genome replication; and *cap*, which encodes four proteins required for capsid formation, namely VP1, VP2, VP3, and assembly activating protein (Sonntag, Schmidt, and Kleinschmidt, 2010; Trempe and Carter, 1988).

AAV infection efficiency on human lymphocytes and their precursors has been examined (Gardner et al., 1997; Horster et al., 1999; Mendelson et al., 1992; Ponnazhagan et al., 1997; Schuhmann et al., 2010; Song et al., 2013a; Song et al., 2013b; Srivastava, 2008; Veldwijk et al., 2010; Zhang and Fuleihan, 1999). AAV2 transduction of primary human T lymphocytes showed significant donor variability with efficiencies ranging from less than 1% to over 50% (Horster et al., 1999; Ponnazhagan et al., 1997). A recent survey on transduction efficiencies of nine naturally occurring AAV serotypes plus one engineered type showed the overall efficiency on immortalized Jurkat T cells (similar to the H9 T cells used in the current study) was 2% or less for 8 of the 10 serotypes. Only AAV1 and AAV6 scored above 2% with efficiencies of only 29% and 20%, respectively, and very high multiplicities of infection (100,000) were required to achieve these efficiencies (Ellis et al., 2013).

With regard to primary stem cells, serotypes 1-10 of AAV were shown to transduce CD34+ hematopoietic stem cells with efficiencies up to 26% (Maguire et al., 2010; Schuhmann et al., 2010; Song et al., 2013a; Song et al., 2013b; Veldwijk et al., 2010; Wang et al., 2015). An enrichment scheme involving fluorescence-activated cell sorting or magnetic beads was used to achieve greater than 85% of targeted gene integration for AAV6 in CD34+ hematopoietic stem cells, as compared with 4-10% efficiency without enrichment (Dever et al., 2016). While this new discovery is promising, *ex vivo* enrichment may not be feasible for many gene transfer applications, and significantly improved AAV vectors are still needed to meet the required therapeutic index for direct *in vivo* applications.

Since the AAV capsid is a primary determinant of transduction, altering and engineering the capsid could conceivably overcome many of the existing transduction and targeting issues, including binding, entry, endosomal escape, and trafficking (Buning et al., 2008; Michelfelder and Trepel, 2009). However, due to the molecular and cellular complexity of each step, rational design of improved AAV-based therapeutics is challenging. For instance, several investigators have attempted to re-target the AAV capsid through the insertion of candidate receptor binding peptides (Perabo et al., 2003; Perabo et al., 2006) and peptides derived from phage libraries (Muller et al., 2003). Viral capsids have also been modified with antibodies (Bartlett et al., 1999) or fusion proteins (Ponnazhagan et al., 2002). Although these approaches have enjoyed some success in re-targeting AAV, they can be accompanied by reductions in titer, and these approaches do not address pre- or post-binding barriers to infection.

Another way to re-target AAV would be to select for mutant capsids that are better able to recognize specific cell surface changes that occur under certain disease conditions. In the case of HIV-1 infection, specific cell surface changes are known to occur upon HIV-1 infection. These surface changes could be exploited to develop a targeted gene therapy vector carrying an anti-HIV-1 payload. For example, the viral envelope protein gp120 is expressed on infected cell membranes, while the viral receptors CD4, CXCR4, and CCR5 are down-regulated (Chenine, Sattentau, and Moulard, 2000; Choi et al., 2008; Hoxie et al., 1986). Major histocompatibility complex class I and II molecules and several cluster of differentiation (CD) antigens, including CD2, CD3, CD5, and CD8 (Meerloo et al., 1992; Stevenson, Zhang, and Volsky, 1987), are also down-regulated. Up-regulation of several CD antigens has also been observed, including CD11a, CD54, and CD63 (Meerloo et al., 1992; Meerloo et al., 1993). These cell surface changes were documented in HIV-1-infected H9 cells, the target cell of the current study (Meerloo et al., 1992; Meerloo et al., 1993).

In general, proteomic analysis of plasma membrane proteins of T cell lines latently infected with HIV-1 revealed the presence of 17 different proteins not found on uninfected cells. Of these, 47% were integral membrane proteins, 18% were membrane-associated proteins, and 35% were cytoplasmic- or organelle-associated proteins (Berro et al., 2007). In addition to the novel proteins, surface glycosylation changes have also been documented in HIV-1-infected T cells, including decreases in sialylation, increases in core 2 O-glycans, and elongation of lactosamine sequence (Lanteri et al., 2003). These cell surface changes, including the expression of HIV-1 gp120 protein on the surface of the cells, could be exploited for targeting AAV to HIV-1-infected cells for research or clinical purposes.

HIV-1 proteins have been used to target cells infected by HIV-1 itself. *Rabies virus, Vesicular stomatitis virus*, and *Murine leukemia virus* were all pseudotyped with CD4 and CXCR4 proteins and shown to bind successfully to the gp120 protein on the surface of HIV-1-infected cells (Mebatsion et al., 1997; Schnell et al., 1997; Somia et al., 2000). Cell killing and reduction of HIV-1 production was demonstrated in the *Vesicular stomatitis virus* system by taking advantage of the natural lytic ability of the virus (Schnell et al., 1997).

The current paper describes the application of a directed evolution approach to select for AAVs that are better able to target HIV-1-infected or uninfected H9 T cells. Reported here is the successful use of this new method to isolate novel AAV capsids.

2. Methods

2.1 Cells

The H9 T cell line, hereafter referred to as H9 cells, was derived from a single cell clone of a specific HUT 78 cell line, HT (Mann et al., 1989; Popovic, Read-Connole, and Gallo, 1984a; Popovic et al., 1984b). HUT 78 cells were derived originally from the peripheral blood of a patient with Sezary syndrome (Gazdar et al., 1980). The H9 cell line was obtained from the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH: H9 from R. Gallo). The 293T cell line is a highly transfectable derivative of the human embryonic kidney cell line, 293 (DuBridge et al., 1987; Pear et al., 1993) and was obtained from the American Type Culture Collection (Manassas, VA). The 293T cell line expresses the SV40 T antigen and contains an insertion of the E1A and E1B adenovirus sequences into chromosome 19 at 19q13.2 (Kovesdi and Hedley, 2010).

The H9 cell line was maintained in RPMI 1640 medium supplemented with 8% heat-inactivated fetal calf serum, and the 293T cell line was maintained in Dulbecco's Modified Eagle's Medium with 10% heat-inactivated fetal calf serum. Both cell lines were also supplemented with 2mM L-glutamine, penicillin (100 U/ml), streptomycin (100 mg/ml). Cell cultures were split at ratios of 1:9 (H9) and 1:8 (293T) twice per week and grown at 37°C and 5% CO_2. The medium, serum, and cell culture supplements were obtained from ThermoFisher.

Cell viabilities were determined using 0.4% trypan blue solution to measure the number of cells capable of excluding the dye.

A chronically infected H9 cell line was established that maintained long-term viability and expression of HIV-1. The H9 cells were chosen because they were pivotal to the discovery of HIV-1, have been used as producer line for HIV-1 ever since its discovery, and have been very well characterized over the past decades. Cells were infected with HIV-1 at a multiplicity of infection (MOI) of 500. After an initial crisis phase of cytopathic effects and cell death, a stable cell line emerged by the eighth passage with a viability of approximately 90%, similar to the parental H9 line. One hundred percent of the cells in this cell line, designated H9-HIV, were HIV-1-positive and produced over 100 ng/ml of HIV-1 p24 protein on a continual basis but otherwise appeared similar to the uninfected H9 cells as viewed under the phase contrast microscope. A seed stock of H9-HIV cells was frozen in liquid

nitrogen, and thawed cells were used between passages 5 and 20 for all experiments. A total of 2.5×10^5 total cells plated at a density of 5×10^5 cells/ml in 48-well plates were used for the biological selection experiments. H9 and H9-HIV cells plated under these conditions showed a doubling time of 40 h.

2.2 Virus

The T-tropic HIV-1 strain HTLV-III_B/H9 was derived from the peripheral blood of a patient with AIDS (Popovic et al., 1984a; Popovic et al., 1984b) and was obtained from the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH: HTLV-III_B/H9 from R. Gallo). The stock of HIV-1 was prepared from infected H9 cells on day 10 post-infection. On the day prior to stock preparation, infected culture medium was completely changed, such that virus stocks contained freshly made virions. Infected cells were centrifuged at 1430 x *g* for 10 min in sealed GH 3.8 rotor buckets of a Beckman GS-6R tabletop centrifuge (Fullerton, CA). Culture supernatants were filtered through a 0.22-μm pore size Steriflip-GP filter unit (Millipore, Bedford, MA), and filtered virus stocks were stored in aliquots at liquid nitrogen vapor phase (approximately -150°C). HIV-1 p24 was quantified using the RETROtek HIV-1 p24 Antigen ELISA from Zeptometrix Corporation (Buffalo, NY).

The AAV library consisted of approximately 10^7 variants generated by a combination of error-prone PCR mutagenesis, peptide insertion, and capsid shuffling (Koerber et al., 2006; Li et al., 2008; Muller et al., 2003). An overrepresentation of AAV2 was present in the library since the mutagenesis and peptide insertions were performed with the AAV2 serotype. The number of variants was determined by quantifying the number of independent bacterial transformants after cloning the original library. Serotypes 1, 2, 4-6, 8, and 9 were chosen for the shuffling because they have been shown to have a diverse range of gene-delivery properties. The AAV library was packaged by using 70 ng of a plasmid encoding the full length AAV genome (psub2cap2) and 25 μg of a plasmid encoding adenovirus helper genes (pHelper).

Wild-type human adenovirus type 5 was obtained from the Viral Vector Core Facility, University of Iowa (Iowa City, IA).

Wild-type AAV2 was prepared by transfecting 293T cells with psub2cap2 and pHelper in a 1:1 ratio.

Recombinant AAVs were made using the *Hind*III and *Not*I restriction sites to replace the wild-type AAV2 *cap* gene with variant *cap* genes that emerged from the H9 selection. The recombinant AAVs were then produced in 293T cells transfected in a 1:1:1 ratio with plasmids encoding: 1) an AAV vector genome; 2) the AAV *rep* and *cap* genes; and 3) a plasmid containing adenovirus helper genes as described previously (Xiao, Li, and Samulski, 1998). The AAVs were purified by iodixanol gradient and were quantified by qPCR to obtain genomic titers.

2.3 Biological selection of AAV

For each of the six rounds of selection, cells were split 24 h prior to use with a complete change of media to ensure cells were in growth phase on the day of infection. Cells were washed three times with PBS pH 7.2. A total of 2.5×10^5 cells were used for the initial infection with the AAV library at a MOI of 10^4. Subsequent rounds of selection used an MOI of 10^2. The reduction in MOI points to the overall strategy for the biological selection in which one starts with a large MOI and then increases stringency in later rounds of selection in order to yield higher specificity for the successful AAVs.

For each such round, cells were infected for 1 h at 37°C, washed twice with culture medium (RPMI 1640 medium supplemented with 8% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin), resuspended in 0.5 ml of medium, and placed in 48-well plates to yield a final cell density of 5.0×10^5 cells/ml. Cells were then incubated for 48 h at 37°C and 5% CO_2. At 48 h post-infection, cells were infected with wild-type adenovirus 5 at an MOI of 10^3 to enrich for the population of AAV variants that were able to infect successfully. Cells were again resuspended in 0.5 ml of medium and placed in 48-well plates for an additional 24 h. Total cellular DNA was then isolated at 72 h post-infection using the DNeasy Blood and Tissue Kit.

For the final three rounds, a negative selection step was performed in the protocol. Cells were incubated for one h at 37°C with the opposing cell line, and the supernatant was then used to infect the cell line of choice for an additional one h.

In total, six rounds of biological selection were performed in which AAV variants were used to infect fresh H9 and H9-HIV cells. Total cell DNA was isolated from the infected cells after each of the six rounds, and the *cap* genes were cloned to create new libraries for subsequent rounds of selection. This process of cloning the *cap* genes for subsequent rounds of selection was performed to enrich for the population of AAV variants that were able to infect successfully. A small number of clones were chosen for sequencing after each round by the Sanger method of sequencing.

2.4. Quantitative PCR

For viral genome quantification, cells were lysed and total cell DNA was purified using the DNeasy Blood and Tissue kit according to the manufacturer's instructions (QIAGEN, Valencia, CA). Quantitative PCR (qPCR) was performed using SYBR Green with low ROX (Quanta) in a Stratagene Real Time PCR System (Agilent Technologies) using primers against the AAV vector and *glyceraldehyde 3-phosphate dehydrogenase* (GAPDH) as an internal standard control. The relative expression of target genes was quantified by comparative Ct analysis using Mx4000p V5 software for data analysis. Each individual qPCR reaction was performed in duplicate. Primers used were: AAV-F-5'-ACGTAAACGGCCACAAGTTC-3' and AAV-R-5'-AAGTCGTGCTGCTTCATGTG-3'; GAPDH-F-5'CACCCTGTTGCTGTAGCCAAA-3'; GAPDH-R-5'-CAACAGCGACACCCACTCCT-3'. The qPCR efficiencies were comparable for both primer sets in the range of -3.0 to -3.4.

2.5 Computer and statistical analyses

All graphs were drawn and all statistical analyses were performed using Sigma plot version 11.0 (Systat Software, Inc., San Jose, CA). Images were processed for figure layout in Photoshop CC 2017 (Adobe Systems, Inc., San Jose, CA). Tests of statistical significance were performed by one-way analysis of variance (ANOVA). Post-hoc testing was performed using the Holm Sidak method. An alpha value of 0.05 was used for all statistical analyses.

3. Results

3.1 Biological selection strategy

A directed evolution approach was implemented to select for AAV variants selective for H9 or H9-HIV cells (Fig. 1). A library of 10^7 AAV variants with mutagenized *cap* genes was packaged and used to infect either H9 or H9-HIV cells. For the first three rounds of positive selection (Fig. 1A), H9 and H9-HIV cells were infected with AAV and allowed to grow for three days with help from wild-type adenovirus during the last day. The AAV *cap* genes were re-cloned and packaged for subsequent infections to enrich the selected virus population for subsequent rounds. Sequence analysis was performed on the *cap* genes after every selection to assess sequence convergence. After the first three rounds of selection, the sequences appeared to be converging, so the isolated *cap* genes were subjected to error-prone PCR mutagenesis to diversify the pool, followed by three more rounds of selection that each included a negative selection step (Fig. 1B). For the negative selections, virus was pre-incubated with the “opposite” cell line to remove nonspecific AAVs and then transferred back to the cell line of interest. That is, AAVs that resulted from the three rounds of selection on H9-HIV cells were negatively selected via pre-incubation with H9 cells followed by infection of H9-HIV cells with the resulting supernatant, or vice versa starting with H9-HIV cells.

3.2. Cell numbers and viabilities remained high during biological selection

We tracked absolute cell counts and viabilities during each round to ensure that we had adequate numbers of cells throughout our selection process. At both the 48 and 72 h time points, absolute cell counts averaged over 8×10^5 total cells with greater than 90% viability at 48 h and greater than 80% at 72 h. The 72 h time point showed slightly less viability as compared to the 48 h time point due to the wild-type adenovirus infection over the final 24 h of the experiment. The addition of wild-type adenovirus did not significantly affect cell viability at 24 h ($p=0.319$) but did reduce viability by 72 h ($p<0.0001$), as determined across multiple rounds by one-way ANOVA. All rounds of infection were performed similarly, and representative data are shown in Fig. 2A-D. As expected, the doubly and triply infected cultures showed lower cell counts and percent viabilities as compared to uninfected controls. At both time points, the H9-HIV cells produced HIV-1 (as measured by p24 production) in amounts equal to uninfected (mock) cells, despite being infected with two additional viruses (Fig. 2E and F). Thus, AAV and adenovirus did not inhibit HIV-1 production.

3.3 Novel AAV variants emerged after six rounds of selection

After six rounds of selection as described above, including three rounds of negative selection at the end, a small number of AAV variants emerged that were all based on the AAV2 backbone. The variants were found to infect both H9 and H9-HIV cells. Four variants representative of the mutations and peptide insertions present after 6 rounds of selection were chosen for further study (Fig. 3). One of the variants, H9-5C (green horizontal bar), emerged from selection on H9 cells, while the other three variants, HIV-2B, 2C, and 4C (red horizontal bars), arose from selection on H9-HIV cells. The H9-5C, HIV-2B, and HIV-4C variants all contained distinct 10-mer peptide insertions in a key loop on the exterior capsid between amino acids 587 and 588, a position present in all three wild-type capsid proteins (VP1, VP2, and VP3; blue horizontal bars). In addition, H9-5C and HIV-4C both had a conservative V708I mutation. Variants HIV-2B and HIV-2C shared a non-conservative E67A mutation, while variants HIV-2C and HIV-4C shared a conservative S207G change. Finally, variants HIV-2B, HIV-2C, and HIV-4C each contained a different, conservative, single point mutation within the VP3 region (I698V, Q598L, and I648V, respectively) (Fig. 3; numbering based on the parent AAV2 VP1 sequence without the peptide insertion).

3.4 Selected AAV variants showed increased cell-association by qPCR analysis

Variant capsids were packaged with an AAV vector and used to infect H9 and H9-HIV cells. Since the mutants that emerged from selection were based on the AAV2 serotype, this serotype was included in the qPCR experiment as a control. Compared to AAV2, variant HIV-2B showed a 9-fold increase in the relative number of cell-associated AAV genomes after infection of H9 (Fig. 4A) and a 4-fold increase after infection of H9-HIV cells (Fig. 4B). Upon infection of H9-HIV cells, variant HIV-4C showed a 3-fold increase in the number of genomes as compared to AAV2 (Fig. 4B). These results were statistically significant by one-way ANOVA, $F(5, 18) = 28.573$, $p < 0.001$ (for H9 cells) and $F(5, 18) = 48.695$, $p < 0.001$ (for H9-HIV cells). Post-hoc testing revealed statistically significant increases in cell-associated viral DNA for HIV-2B on both H9 and H9-HIV cells ($p < 0.001$) and for HIV-4C on H9-HIV cells ($p < 0.001$).

4. Discussion

In this study, a triple infection of H9 cells was performed to establish a system for selecting AAV variants with increased tropism for these cells. The results showed that all three viruses (AAV, adenovirus, and HIV-1) replicated in the presence of each other while maintaining sufficient cell viability to conduct selections (Fig. 2). The triply infected cells showed the greatest reduction in cell number, presumably due the cytotoxic effects of the wild-type adenovirus infection, which was used to enrich for successful AAVs in each round of selection. For a final gene delivery application, the wild-type adenovirus would no longer be needed because a highly transducing AAV would have already been chosen as the end product of a selection process.

Harnessing this system, a directed evolution approach was used to select for novel AAV capsids over six rounds of infection on H9 and H9-HIV cells, involving three rounds of positive selection followed by three rounds of coupled negative/positive selection (Fig. 1).

Four mutants were chosen after the final selection step. Two of these mutants (HIV-2B and HIV-4C) were shown to have improved tropism for H9-HIV cells, and one of these two (HIV-2B) also had improved tropism for the parental H9 cells as evidenced by significant increases in cell-associated viral genomes measured by qPCR (Fig. 4). The fact that the selections converged on a small number of variants is not unusual. For example, one previous report on AAV selection described the isolation of a single, unique point mutation that exhibited enhanced binding to the apical surface of human airway epithelia as well as improved gene transfer in those cells (Excoffon et al., 2009). The observation that HIV-2B showed significant increases in cell-associated DNA on both H9 and H9-HIV cells (as compared with AAV2; Fig. 4) may indicate that this AAV variant utilizes a shared mechanism of cellular targeting between H9 and HIV-H9 cells. The two variants were used to transduce primary CD4+ T cells and showed an efficiency equal to that of parental AAV2 (data not shown).

In establishing and characterizing the system, we found that HIV-1 p24 production was not inhibited in H9 cells during co-infection (Fig. 2E and F), in contrast with previous reports indicating that AAV2 Rep protein may inhibit HIV-1 (Antoni et al., 1991; Mendelson et al., 1992; Oelze, Rittner, and Sczakiel, 1994; Rittner et al., 1992). These previous studies introduced the *rep* gene by microinjection or transfection, making it difficult to compare results with the current study. One of the studies used lower MOIs for both AAV (100 to 200 infectious units) and adenovirus helper (20 infectious units) as compared to the current study, and the duration of the experiment was longer (one week as compared to 24 h in the current study) (Mendelson et al., 1992). These experimental differences may likely explain the discrepancies between previous and current results with regard to inhibition of HIV-1 by the Rep protein.

One of the previous studies investigating Rep inhibition of HIV-1 used infectious viruses and described a triple infection of HUT78 cells with HIV-1, AAV2, and human adenovirus 2 (HUT78 is the parental line of H9 cells) (Mendelson et al., 1992). In this study, there was no reduction in HIV-1 production during infection by adenovirus alone, a 16% reduction by AAV alone, and a 51% reduction by both AAV and adenovirus, as measured by reverse transcriptase assay (Mendelson et al., 1992). AAV and adenovirus could exert opposing effects on HIV-1 during a triple infection, since adenovirus has been shown to activate HIV-1 (Antoni et al., 1991) whereas AAV has been shown to inhibit it via binding of viral proteins to the HIV-1 long terminal repeat (Oelze et al., 1994; Rice and Mathews, 1988; Rittner et al., 1992). Different serotypes of AAV may also exert different effects on HIV-1 replication, and this possibility could be explored further in future studies.

Although the original AAV library contained shuffled capsids from seven different serotypes of AAV (types 1, 2, 4–6, 8, and 9), the mutants that emerged after six rounds of selection were all based on the AAV2 serotype with a 98.6-99.9% sequence identity at the amino acid level. The fact that the selected variants had an AAV2 backbone does not necessarily mean that wild-type AAV2 is good, but rather only that small changes to a parental serotype like AAV2 are sufficient to positively shift tropism.

It is also important to consider the fact that DNA shuffling generally leads to smaller numbers of functional variants because of the modular nature by which the proteins are assembled; shuffled variants will only be functional if the structural modules are maintained. This is particularly important for a viral capsid since it is a multifunctional protein. As such, the shuffled clones must maintain functionality in multiple areas of the protein surface. All of the different classes of libraries (point mutants, peptide insertions, and shuffled capsids) were mixed together and allowed to compete with each other in this study. In previous studies, point mutants or peptide insertion variants have been observed to outcompete shuffled variants (Steines et al., 2016). However, one previous study showed positive selection for a shuffled variant with increased tropism for human airway epithelial cells, thus providing evidence that shuffled libraries are functional (Excoffon et al., 2009).

Wild-type AAV2, which binds heparan sulfate proteoglycans as its primary receptor, has a natural tropism for liver, kidney, neurons in the central nervous system, and retinal pigment epithelium in the eye (Burger et al., 2004; Grimm and Kay, 2003; Kwon and Schaffer, 2008; Wu, Asokan, and Samulski, 2006). Each of the point mutations identified in the current study has been observed by others in previous selections (Koerber et al., 2009; Steines et al., 2016) but in different combinations and without the peptide insertions, raising interesting mechanistic questions for future investigation. These mutants could be used to explore mechanisms of AAV transduction to enable better vector development, as well as to create new AAV libraries for further selection on other cells, including primary and latent T cells.

5. Conclusion

The experiments described herein were aimed at establishing a system for selecting AAV variants on HIV-1-infected and uninfected H9 T cells. This initial, proof-of-concept study used a cell line to develop the method for this type of selection with three replicating viruses (AAV, adenovirus, and HIV-1). Future studies will be aimed at performing the described selection process on other cell types, such as primary T cells, which have additional technical challenges, such as cell viability and donor variability.

It is important to note that the entire AAV selection process (all six rounds or more) must be performed on the target cell of final interest. It would be highly unlikely for an AAV variant to be successful on primary T cells if it had been previously selected on an immortalized cell line, such as the H9 line described here. The AAV variants selected in the current study were not expected to translate directly into an *in vivo* application. Rather, they were intended to provide evidence that unique AAVs could be isolated in this way.

Using AAV to target HIV-1-infected cells offers a potential research tool or gene therapy approach aimed at delivering an anti-HIV-1 therapeutic molecule, such as a small interfering RNA molecule or a genome editing system (Bobbin, Burnett, and Rossi, 2015; Limsirichai, Gaj, and Schaffer, 2016; Yin et al., 2017). Alternatively, isolation of an AAV variant that is better able to transduce non-HIV-1 infected T cells could be used potentially to protect them from HIV-1 infection as part of a prophylactic strategy. The new method may also be applicable to other T cell disorders, such as cancer, autoimmunity, and other non-HIV-1 immune deficiencies.

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Highlights

- A novel AAV directed evolution approach to target parental and HIV-1-infected cells
- Triple infection of H9 T cells with AAV, adenovirus, and HIV-1 was successful
- Novel AAV capsids were isolated that show increased tropism toward the target cells

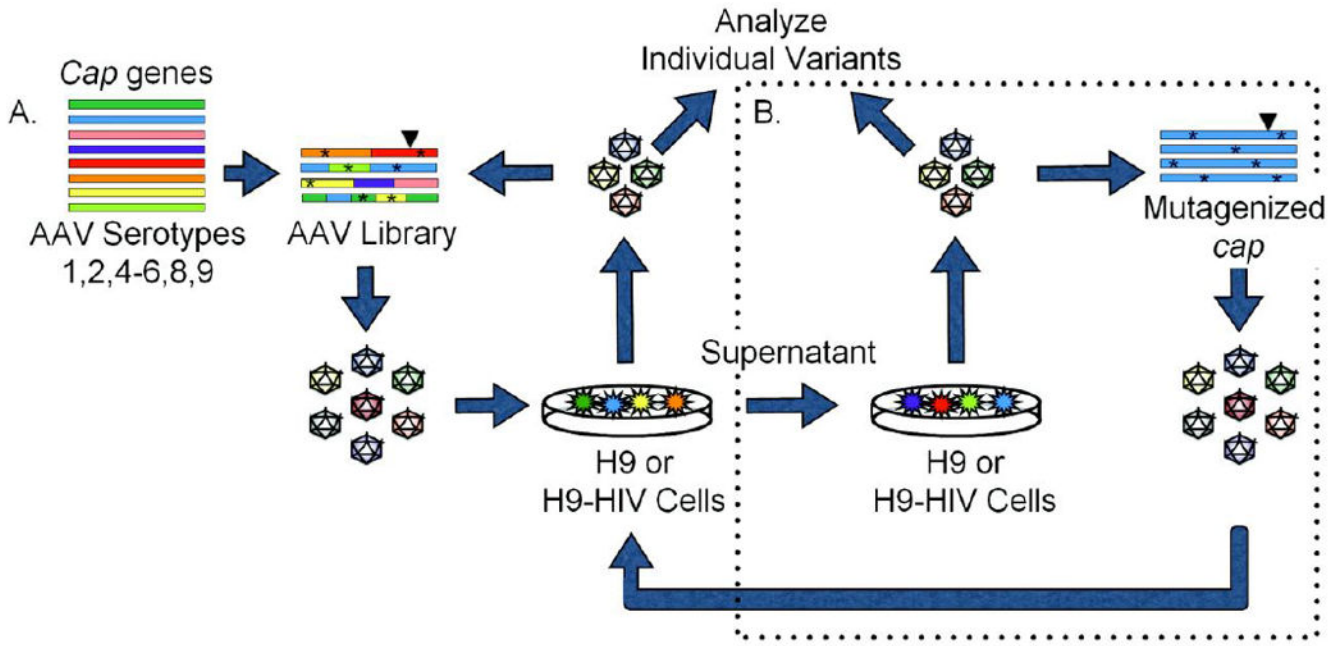

Figure 1. Selection scheme for AAV variants

(A) A library of AAV mutants was packaged and used to infect either H9 or HIV-infected H9 cells. Wild-type adenovirus was added as a helper virus to induce replication of AAV that had infected the cells, and the resulting AAV clones were recovered by PCR amplification and cloning their *cap* genes, which were then re-packaged for two subsequent infections. (B) After three selections, isolated *cap* genes were subjected to error-prone mutagenesis and three additional rounds of selection were performed including a negative selection step, whereby virus was pre-incubated with the opposite cell line before infecting the cell line of interest. Asterisks (*) represent point mutations; Triangles (▼) represent peptide insertion.

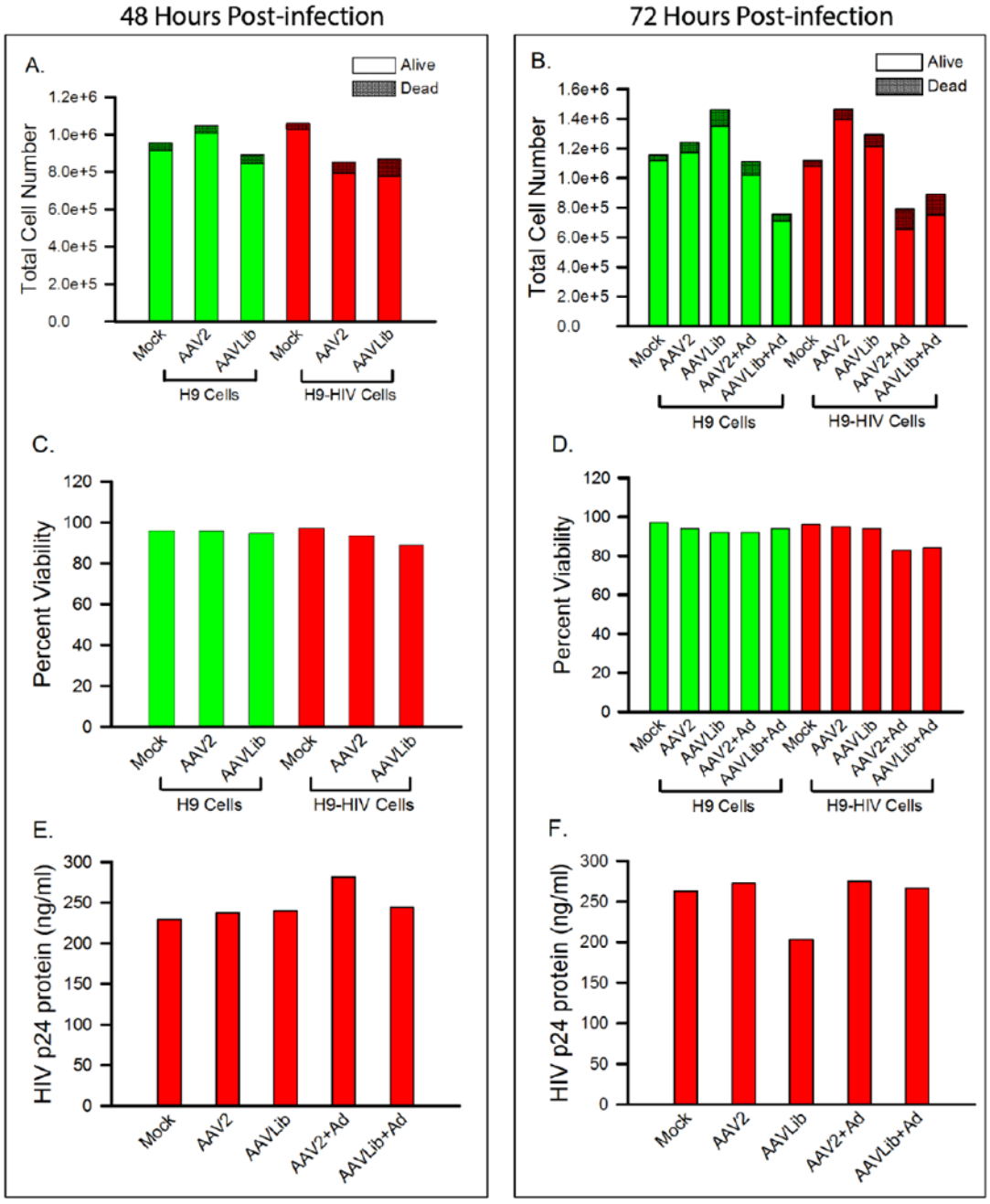

Figure 2. Cell counts, viabilities, and p24 production at 48 and 72 h post infection with AAV

(A) Absolute cell counts at 48 h post-infection and (B) absolute cell counts at 72 h post-infection. (C) Viabilities at 48 h post-infection and (D) viabilities at 72 h post-infection. (E) HIV p24 production at 48 h post-infection and (F) HIV p24 production at 72 h post-infection. Mock: cells without virus; AAVLib: AAV library of variants; Ad: wild-type adenovirus.

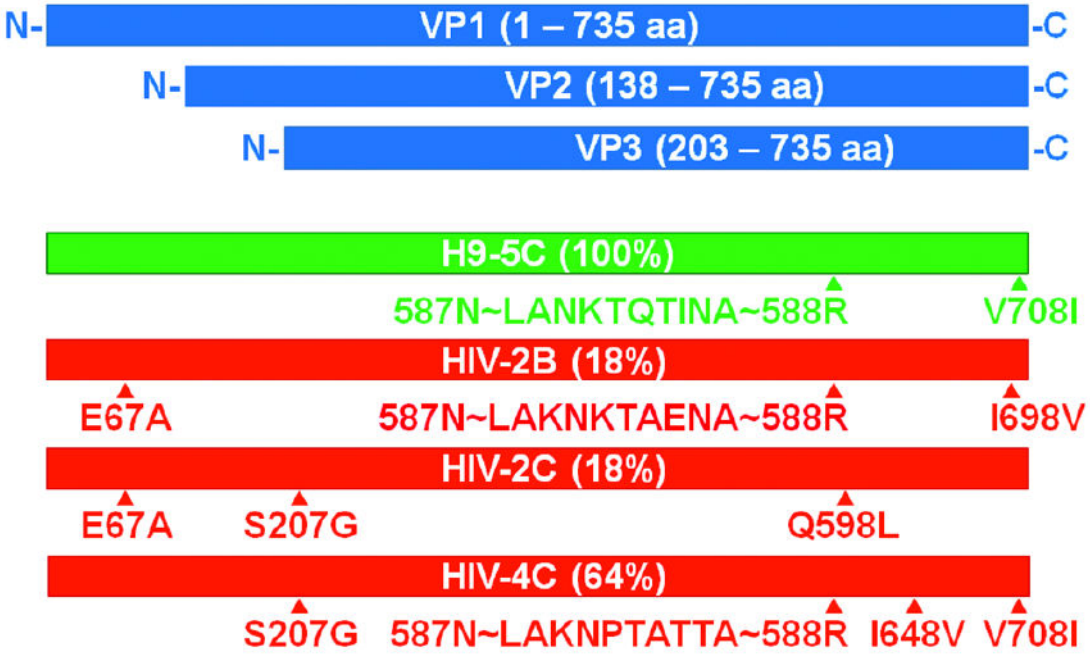

Figure 3. Selected AAV variants after six rounds of selection

Wild-type AAV encodes three viral capsid proteins, (VP1, VP2, and VP3; blue), in the stoichiometric ratio of 1:1:10 through the use of alternative splice sites and nonconventional translational initiation codons. After six rounds of selection, the AAV library converged to a small number of sequences. One predominant sequence for H9 cells (green) and three predominant sequences for H9-HIV cells (red) are shown with percentages indicating prevalence within the population. The majority of sequence changes are located in the region encompassing all three of the viral capsid proteins. Triangles (▲) represent locations of point mutations or peptide insertions. Letters represent amino acid code. The letter before the number represents the wild-type amino acid, while the letter after the number represents the new amino acid. Peptides were inserted between amino acids 587 and 588 of the wild-type AAV 2 capsid protein. The first two and last amino acids were invariant amongst the peptides due to restriction site sequence. The figure is drawn approximately to scale.

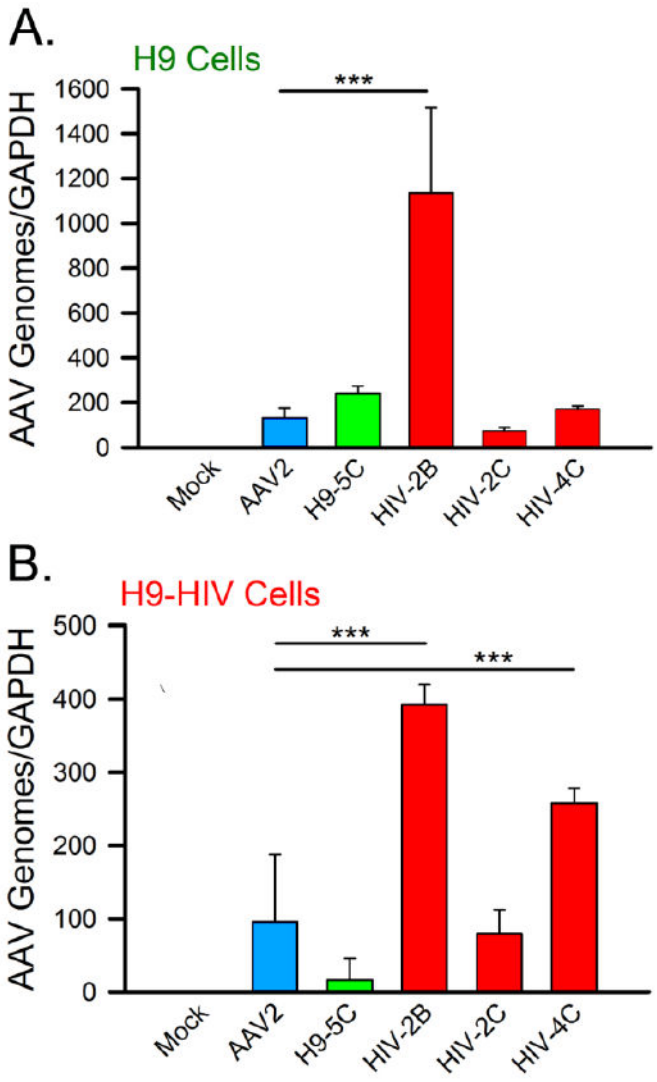

Figure 4. Number of cell-associated genomes for selected AAV variants

Specific AAV variants were packaged and used to infect either (A) H9 or (B) H9-HIV cells. qPCR was performed on total cell DNA isolated from the infected cells or control cells with normalization against the GAPDH gene. The bar graphs are color-coded as follows: blue = AAV2; green = variant isolated from H9 cells (H9-5C); red = variants isolated from H9-HIV cells (HIV-2B, HIV-2C, and HIV-4C). Mock: cells without virus. GAPDH: glyceraldehyde 3-phosphate dehydrogenase housekeeping gene for qPCR normalization. The horizontal bars and triple asterisks represent statistical significance between the designated conditions at a level of $p < 0.001$.