Kinetics of Adeno-Associated Virus Serotype 2 (AAV2) and AAV8 Capsid Antigen Presentation *In Vivo* Are Identical

Yi He,^{1,2,*} Marc S. Weinberg,^{2,*} Matt Hirsch,² Mark C. Johnson,³ Roland Tisch,³
R. Jude Samulski,^{2,4} and Chengwen Li^{2,5}

Abstract

Adeno-associated viral (AAV) vectors 2 and 8 have been used in clinical trials for patients with hemophilia, and data suggest that the capsid-specific CD8⁺ T cell response has had a negative impact on therapeutic success. To date the pattern of capsid cross-presentation from AAV2 and AAV8 transduction *in vivo* has not been elucidated. Previously, we have demonstrated that an engineered AAV2 virus carrying the immune-dominant SIINFEKL peptide in the capsid backbone was indistinguishable from wild type with respect to titer, tropism, and the ability to induce capsid-specific CD8⁺ T cell responses *in vivo*. In this study, we used the same strategy to engineer an AAV8 vector and demonstrated that antigen from SIINFEKL peptide-integrated AAV8 capsid was effectively presented via either plasmid transfection or AAV8 transduction *in vitro*. The tissue tropism and transgene expression kinetics of the engineered AAV8 vector *in vivo* were identical to that of wild-type AAV8. Animal studies show that capsid antigen presentation from AAV transduction was dose dependent, and more importantly, the proliferation of capsid-specific CD8⁺ T cells had similar kinetics (detectable before 30 days and undetectable after 40 days) for both AAV2 and AAV8 vectors. Elucidation of the kinetics of capsid antigen presentation from AAV transduction by various serotypes provides new insight into the potential impact CD8⁺ T cells can have during clinical trials and may help with rational design of effective strategies to prevent capsid-specific CD8⁺ T cell-mediated elimination of AAV-transduced target cells.

Introduction

ADENO-ASSOCIATED VIRUS (AAV) is a single-stranded DNA virus with a genome composed of the *rep* and *cap* genes flanked by inverted terminal repeats (ITRs). One AAV virion contains three capsid proteins—VP1, VP2, and VP3—at a ratio of 1:1:8. Substitution of the *rep* and *cap* genes with a therapeutic gene generates an AAV vector. AAV serotype 2 (AAV2) was first isolated from adenovirus contamination and has been extensively studied for its virology and as a gene delivery vehicle. Eleven other AAV serotypes and more than 100 AAV variants have been isolated and studied to extend AAV vector tropism with enhanced transduction efficiency for the purpose of gene therapy. AAV vectors have been used

in more than 80 clinical trials, with no serious AAV-related toxicities. Promising results have been achieved in two clinical trials in patients with Leber disease or hemophilia (Manno *et al.*, 2006; Maguire *et al.*, 2008, 2009; Simonelli *et al.*, 2010; Nathwani *et al.*, 2011): For Leber disease, after delivery of an AAV2 vector encoding RPE65 into the retina, patients' vision was restored (Maguire *et al.*, 2008, 2009; Simonelli *et al.*, 2010). For patients with hemophilia B, an AAV2 vector encoding human coagulation factor 9 (F9) was delivered via an artery, resulting in 10% blood level detection of F9; unfortunately, therapeutic levels of F9 lasted only 4 weeks, and then returned to the baseline level, concurrent with elevation of liver transaminases. No CD8⁺ cytotoxic T lymphocyte (CTL) response to F9 protein was detected, but peptides from the

¹Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin 300020, China.

²Gene Therapy Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599.

³Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599.

⁴Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599.

⁵Department of Pediatrics, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599.

*Y.H. and M.S.W. contributed equally.

AAV2 capsid induced lymphocyte activation, which suggested that capsid-specific CTLs destroyed the AAV2-transduced liver cells and resulted in the failed treatment (Manno *et al.*, 2006). In a second hemophilia clinical trial, an AAV8 vector was used to deliver F9 into the liver via peripheral vein administration. A capsid-specific CTL response was detected in all patients at various time points after AAV delivery (Nathwani *et al.*, 2011), and two patients receiving a high vector dose had liver transaminase elevation coincident with detection of an AAV8 capsid-specific T cell response. The clinical findings that a capsid-specific T cell response is elicited from AAV vector transduction was not previously observed in preclinical studies (Nathwani *et al.*, 2011). Later studies, however, showed that indeed a capsid-specific CTL response could be mounted from AAV vector transduction via muscular injection or immunization with AAV vector-pulsed dendritic cells (Chen *et al.*, 2006; Li *et al.*, 2007; Wang *et al.*, 2007). Clinical data indicate that the capsid-specific CTL response is dose dependent, such that a stronger CTL response results from higher AAV particle delivery (Manno *et al.*, 2006; Nathwani *et al.*, 2011). The capsid CTL response was elicited regardless of administration route (Manno *et al.*, 2006; Mingozzi *et al.*, 2009; Nathwani *et al.*, 2011), that is, whether the vector was delivered by muscular injection (Mingozzi *et al.*, 2009), artery injection (Manno *et al.*, 2006), or peripheral vein injection (Nathwani *et al.*, 2011).

To mimic the findings from clinical trials, AAV2 vectors have been used to test the kinetics of antigen presentation in animal models. Such studies have indicated that capsid-specific T cell activation is observed at early time points after vector application (H. Li *et al.*, 2011). Among 12 serotypes and more than 100 variants, AAV serotype 8 is considered to be the most efficient for liver transduction (Gao *et al.*, 2006; Wu *et al.*, 2008; C. Li *et al.*, 2011; Wang *et al.*, 2012). Furthermore, peak levels of transgene expression are obtained significantly sooner with AAV8 as compared with AAV2 packaged vectors (Wu *et al.*, 2008). To determine whether the kinetics of a capsid-specific CTL response *in vivo* differ between AAV2 and AAV8, we adopted a previously used strategy of substituting the capsid HI loop with the chicken ovalbumin (OVA)-derived SIINFEKL peptide to generate an AAV8OVA cassette (Li *et al.*, 2009b). Using the engineered AAV8OVA vector, we found that AAV8 capsid antigen was properly processed and presented on the cell surface with MHC class I molecules to activate OVA-specific lymphocytes either through endogenous production of capsid or by AAV8OVA vector transduction *in vitro*. *In vivo* administration of AAV8OVA induced tropism and kinetics of transgene expression similar to those of wild-type AAV8 vector. Prominently, similar *in vivo* kinetics of capsid antigen presentation were observed for AAV8OVA and AAV2OVA vectors.

Materials and Methods

Constructs

The SIINFEKL immunodominant epitope was cloned into the AAV8 capsid by substitution of the HI loop with the eight-amino acid sequence as described previously for generation of the pXR2/OVA cassette (Li *et al.*, 2009). We first used the PCR primers F1R1-0 and F2-0R2 (Supplementary Fig. S1; supplementary data are available online at www.liebertonline.com/hum), which were designed to in-

troduce the SIINFEKL peptide into the HI loop of the AAV8 capsid, using the pXR8 template to match the OVA peptide positioning with that placed in the AAV2 capsid for pXR2/OVA based on alignment (Supplementary Fig. S1). The PCR products from primers F1R1 and F2R2 were digested with *SalI* and *NotI*, respectively. pXR8/OVA-0 was generated by insertion of digested PCR products into the pXR8 backbone with *SalI* and *NotI* digestion. However, virus production yield from pXR8/OVA-0 transfection was low (data not shown). We tested whether repositioning the swapped peptide downstream by one or two amino acids would improve virus yield. To this end we used primer sets F1R1-1/F2-1R2 and F1/R1-2/F2-2R2 to generate the constructs pXR8/OVA-1 and pXR8/OVA-2, respectively. These two constructs produced higher virus yields, with the highest yield coming from pXR8/OVA-1 and matching the yield from wild-type AAV8 vector (data not shown). So, throughout this study, pXR8/OVA-1 was used to make AAV8OVA vectors. All pXR8/OVA constructs were verified by sequencing. The constructs pTR-CBA-luc and pTR-CBA-AAT are AAV2 TR-containing plasmids encoding the firefly luciferase and human α_1 -antitrypsin (AAT) transgenes, respectively, and were driven by the chicken β -actin promoter (CBA).

Cells and virus

HEK293 and HepG2/H-2K^b were maintained at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/ml), and streptomycin (100 g/ml). AAV virus production was done as previously described, using three-plasmid cotransfection in 293 cells (Xiao *et al.*, 1998). The viral titer was determined by Southern dot blot.

Mice

C57BL/6 mice and BALB/c mice were purchased from Jackson Laboratory (Bar Harbor, ME). OT-1/Rag-1 mice express an H-2K^b-restricted T cell receptor (TCR) specific for the OVA-derived SIINFEKL peptide and were ordered from Taconic Farms (Germantown, NY). All mice were maintained in a specific pathogen-free facility at the University of North Carolina at Chapel Hill (Chapel Hill, NC), and the University of North Carolina Institutional Animal Care and Use Committee approved all procedures.

Antigen presentation assay *in vitro* after pAAV8/OVA transfection or AAV8OVA vector transduction

Activation of CD8⁺ OT-1 T cells, measured by upregulation of CD69 expression via flow cytometry, was used as a readout for the efficiency of antigen presentation of the SIINFEKL-containing capsids after transfection or transduction of AAVOVA plasmids and packaged vectors, respectively. For transfection studies, 293 cells at 70–80% confluency in a 6-well plate were transfected with 3 μ g of each plasmid including pXR8/OVA, pXX6-80, and pCBA-H-2K^b, using polyethylenimine (PEI). After 24 hr, cells were incubated with OT-1 T cells overnight. In transduction studies, 2×10^5 HepG2/H-2K^b cells were incubated with 2×10^{11} particles of AAV vector for 24 hr, and then washed and incubated with OT-1 T cells overnight.

Flow cytometry

Phycoerythrin (PE)-labeled rabbit anti-mouse CD69 (clone H1.2F3; BD Biosciences, San Jose, CA) and a fluorescein isothiocyanate (FITC)-labeled anti-CD8 (clone 53-6.7; BD Biosciences) were used to stain OT-1 T cells for 1 hr at 4°C. Cells were then washed and analyzed by flow cytometry (UNC Chapel Hill Flow Cytometry Core Facility).

Luciferase imaging

BALB/c mice were retro-orbitally injected with 1×10^{11} particles of AAV/luc, and 1 week later, imaging was performed with a Xenogen IVIS Lumina imaging system (Caliper Life Sciences/PerkinElmer, Hopkinton, MA) after intraperitoneal injection of D-luciferin substrate (120 mg/kg; NanoLight Technology, Pine-top, AZ). Bioluminescence image analysis was carried out with Living Image software (PerkinElmer, Waltham, MA).

ELISA for human AAT

The AAT concentration in blood was determined by enzyme-linked immunosorbent assay (ELISA) as described previously (C. Li *et al.*, 2011).

In vivo CTL killing assay

As described previously (Li *et al.*, 2009), 10^7 spleen cells from C57BL mice were incubated with either a high dose (5 M) or low dose (0.5 M) of carboxyfluorescein succinimidyl ester (CFSE) in phosphate-buffered saline (PBS) at room temperature for 12 min. Fetal bovine serum (FBS) was then added to stop CFSE labeling. After washing, CFSE^{high} cells were incubated with OVA SIINFEKL (10 µg/ml). After peptide pulsing, two populations (CFSE^{high} and CFSE^{low}) of target cells were washed and mixed together, and then 10^7 cells of each population were injected into AAV8OVA-immunized mice via the tail vein. Spleen cells were collected 24 hr later and analyzed by flow cytometry. The percent specific lysis was determined by the following formulas: the ratio of recovery of non-peptide-treated control spleen cells to peptide-sensitized spleen cells = (percentage of CFSE^{low} cells)/(percentage of CFSE^{high} cells). The percent specific lysis (%) = $100 \times [1 - (\text{ratio of cells recovered from naive mice} / \text{ratio of cells recovered from infected mice})]$.

In vivo T cell proliferation

C57BL/6 mice received AAVOVA vector via retro-orbital administration. At various time points after AAV injection, CFSE-labeled OT-1 T cells were transferred and T cell proliferation was measured. OT-1 T cells were isolated from spleen and incubated for 10 min at room temperature in PBS with 5 µM CFSE at 2×10^7 cells/ml. The reaction was stopped by the addition of FBS, cells were washed with PBS, and 5×10^6 CFSE-labeled OT-1 T cells were retro-orbitally injected into C57BL/6 mice. On day 10 after OT-1 T cell transfer, spleen cells were harvested and stained with antibodies specific for TCR α -chain V₂ and CD8, or with PE-conjugated H-2K^b/SIINFEKL tetramer. The frequency of proliferating T cells was determined by the percentage of CFSE dilution (Krishnan *et al.*, 2007). The proliferation index (PI) was calculated as the ratio of the total number of cells to the number of cells initially residing in the parent generation.

Statistical analysis

The Student *t* test was used to carry out statistical analysis. $p < 0.05$ was considered statistically significant.

Results

AAV8OVA capsid stimulates CD8⁺ OT-1 T cell reactivity

The AAV8 capsid protein is associated with increased transduction efficiency and broader tissue tropism compared with the AAV2 capsid, based on animal studies (Wu *et al.*, 2008). To study capsid-specific CTL reactivity, we previously established a system in which the AAV2 virus was engineered with an HI loop substitution in the AAV2 capsid, using the chicken ovalbumin immunodominant SIINFEKL peptide sequence (AAV2OVA) (Li *et al.*, 2009). Using this engineered AAV2OVA virus, we demonstrated that incubation of AAV2OVA-transduced H-2K^b-expressing cells with spleen cells from OT-1 mice induced the activation of spleen cells *in vitro*, and administration of AAV2OVA virus elicited a capsid-specific CTL immune response in mice (Li *et al.*, 2009, 2013). To investigate AAV8 capsid antigen presentation and immunogenicity, an AAV8OVA capsid, prepared similarly to AAV2OVA, was engineered by replacing the AAV8 HI loop with the OVA SIINFEKL peptide. First we confirmed that the SIINFEKL epitope was processed and presented, leading to OT-1 T cell stimulation. 293 cells were cotransfected with AAV8OVA plasmid, an H-2K^b expression plasmid, and pXX6-80, which encodes adenoviral proteins to induce capsid expression. The stimulatory capacity of the 293 transfectants was then tested by coculture of the cells with OT-1 T cells and by measuring upregulation of CD69 expression. 293 cells transfected with AAV8OVA plasmid activated OT-1 T cells to a similar extent as AAV2OVA transfectants, whereas cells transfected with a plasmid encoding wild-type AAV8 capsid or mock transfected did not (Fig. 1). Next, we assessed the T cell-stimulatory capacity of HepG2/H-2K^b cells, an H-2K^b-expressing hepatocyte cell line (Li *et al.*, 2013) that were transduced with AAV8OVA packaged vector. Whereas wild-type AAV8 vector-transduced HepG2/H-2K^b cells did not activate OT-1 T cells relative to the no-vector control (0.20 and 0.12% CD8⁺ CD69⁺ cells, respectively), AAV8OVA transduction resulted in an increased percentage of CD8⁺ CD69⁺ T cells (1.29%). However, this frequency was significantly lower than that detected in cultures of AAV2OVA-transduced HepG2/H-2K^b cells (10.89%; Fig. 1). These results indicate that the SIINFEKL peptide in the context of the AAV8 capsid is processed and presented, and stimulates CD8⁺ T cells albeit at a reduced level compared with that resulting from the AAV2OVA capsid.

Similar transduction efficiency and tropism in vivo between AAV8OVA and wild-type AAV8 vectors

To study antigen presentation of the AAV8 capsid, we initially determined whether insertion of the SIINFEKL epitope into the HI loop of the AAV8 capsid altered tissue tropism *in vivo* by monitoring luciferase expression *in vivo* in mice injected with AAV8OVA/luc vector. As shown in Fig. 2A, the pattern of luciferase expression was similar for animals injected with AAV8OVA/luc and wild-type

FIG. 1. AAV8OVA capsid protein stimulates OT-1 T cells *in vitro*. Activation of OT-1 T cells, based on upregulation of CD69 expression, was measured via flow cytometry on coculture with (A) 293 H-2K^b cells transfected with pAAV8OVA or pAAV2OVA plasmids or left untreated (Control), and (B) HepG2/H-2K^b cells transduced with packaged AAV8OVA or AAV2OVA or left untreated.

AAV8/luc. Notably, for both AAV8 vectors the strongest signal was localized to the liver. Next, we injected AAV8OVA/AAT vector intravenously into mice, and measured blood levels of AAT. Similar levels and kinetics of AAT expression over 3 months were detected in mice treated with the AAV8/AAT and AAV8OVA/AAT vectors. Transgene levels peaked within 2 weeks postinjection, remained stable

over 10 weeks, and decreased slightly at week 14 in both groups (Fig. 2B).

T cell response to AAV8OVA capsid in mice

In previous studies, we demonstrated that muscular administration of AAV2OVA vector mounts an OVA peptide-

FIG. 2. AAV8OVA- and wild-type AAV8-packaged vectors exhibit similar transduction profiles. (A) Particles of AAV8OVA/luc or AAV8/luc vector (1×10^{11}) were injected into BALB/c mice and, 1 week later, luciferase expression was monitored by whole body imaging with a 10-sec exposure, using a Xenogen IVIS Lumina system. (B) C57BL/6 mice received intravenously 1×10^{11} particles of AAV8OVA/AAT or AAV8/AAT vector, and at various time points AAT levels in blood were measured by ELISA.

specific CTL response (Li, Hirsch *et al.*, 2009). To determine whether administration of the AAV8OVA vector elicited a capsid CTL response *in vivo*, we administered AAV8OVA/AAV2 vector at a dose of 1×10^{11} particles via muscular injection into C57BL/6 mice. Thirty days after AAV8OVA injection, CFSE-labeled naive syngeneic mouse spleen cells pulsed with OVA SIINFEKP peptide were infused into mice. After 24 hr, the mice were killed and the amount of cells pulsed with OVA peptide was calculated by flow cytometry. As shown in Fig. 3, compared with control mice, no obvious killing of OVA peptide-pulsed cells was observed in mice receiving AAV8 or AAV8OVA vector.

Cross-presentation of AAV capsid antigen is dose dependent *in vivo*

The efficiency of cross-presentation is weak compared with endogenous antigen presentation. Usually high exogenous antigen is required to elicit a strong cellular immune response. In this study, we determined the efficiency of *in vivo* SIINFEKL presentation by measuring the proliferation of transferred OT-1 T cells. To investigate the effects of

the dose of AAVOVA capsid on antigen presentation and OT-1 T cell stimulation, various particle numbers of AAV2OVA vector were injected into C56BL/6 mice. When compared with control mice without AAV2OVA treatment, a dose of 5×10^{10} particles and higher induced OT-1 T-cell proliferation (Fig. 4). However, no proliferation of OT-1 T cells was detected in groups with doses of AAV2OVA less than or equal to 1×10^{10} particles. Similar dose-dependent antigen presentation was observed when using the AAV8OVA vector *in vivo* (data not shown). These data suggest that efficient T cell stimulation occurs only with high doses of AAV vector.

The extent and kinetics of AAV8 capsid antigen presentation *in vivo* are similar to that of the AAV2 capsid

It has been shown that transgene expression gradually increases after AAV2 administration in mice and peaks at week 6, remaining stable long-term after vector injection. Although the epitopes from the AAV2 capsid are cross-presented by antigen-presenting cells (APCs) to elicit a capsid-specific CTL response, it is unclear whether the kinetics of

FIG. 3. No OVA-specific CTL-induced cytotoxic activity was elicited by muscular injection of AAV8OVA vector. AAV8OVA/AAV2 vector (1×10^{11} particles) was injected into the muscle of C57BL/6 mice, and 4 weeks later the mice received via tail vein injection a mixture of C57BL/6 mouse spleen cells labeled with either a high concentration of CFSE (CFSE^H) and pulsed with SIINFEKL peptide, or a low concentration of CFSE (CFSE^L). After 24 hr, single cells were isolated from the spleens and analyzed by flow cytometry to determine *in vivo* CTL-mediated killing. (A) Each percentage represents the *in vivo* CTL-specific killing from one of four mice. (B) Average killing efficiency of OVA-specific CTLs elicited by AAV8OVA muscle transduction in four mice.

FIG. 4. Capsid antigen presentation after AAVOVA transduction is dose responsive *in vivo*. Various doses of AAV2OVA/AAT vector were injected intravenously into C57BL/6 mice and 3 days later, CFSE-labeled OT-1 T cells were transferred. On day 10 after transfer OT-1 T cell proliferation in the spleen was assessed via flow cytometry. (A) Representative flow cytometric histograms. (B) Average T cell proliferation and standard deviation of four mice. (C) Average proliferation index (PI) and standard deviation. ** $p < 0.01$, * $p < 0.05$ compared with control mice without AAV treatment.

antigen presentation of capsid epitopes *in vivo* after AAV vector administration correspond with the dynamics of transgene expression. To address this question, AAV2OVA/AAT vector (2×10^{11} particles) was injected intravenously, and at various time points splenic OT-1 T cells labeled with CFSE were transferred into mice. Ten days posttransfer OT-1 T cell division was measured by flow cytometry. As shown in Supplementary Fig. S2, over days 3–12 and days 21–30, OT-1 T cell division was significantly increased in AAV2OVA/AAT vector-treated (86.73 and 55.63%, respectively) versus control recipients (30 and 24.7%, respectively; $p < 0.01$ and $p < 0.05$, respectively). However, no difference was observed for OT-1 cell proliferation between AAV2OVA and control groups over days 41–50 and days 61–70 ($p > 0.05$). This result indicates that antigen cross-presentation from the AAV2 capsid occurs during the earlier time period after AAV2 systemic administration.

To elucidate the kinetics of antigen presentation from AAV8 transduction *in vivo*, we injected 1×10^{11} particles of AAV8OVA/AAT or AAV2OVA vector into C57BL/6 mice, and assessed proliferation of CFSE-labeled OT-1 T cells transferred at various times after vector treatment. As shown in Fig. 5, the frequency of proliferating OT-1 T cells was significantly increased in mice injected with AAV8OVA between days 3–12 and days 21–30, relative to the control group ($p < 0.05$). Although slightly more cells in division from AAV8OVA application were observed compared with that from control at later time points (days 41–50 and days

61–70), these findings were not statistically significant (Fig. 5B). Furthermore, no marked difference was observed in the level and kinetics of OT-1 T cell proliferation between animals treated with AAV8OVA and AAV2OVA. These results demonstrate that presentation of the SIINFEKL epitope is similar for both AAV8 and AAV2 capsids.

Discussion

In this study we demonstrated through plasmid transfection and *in vitro* virus infection that integration of the OVA immunodominant peptide SIINFEKL into the AAV8 capsid induces antigen processing and presentation. Transgene expression pattern and kinetics subsequent to AAV8OVA vector administration were similar to those of wild-type AAV8 *in vivo*. A functional T cell response against OVA peptide was not observed *in vivo* after AAV8OVA administration. Antigen cross-presentation from AAV2 transduction *in vivo* is dose dependent. Most significantly, similar to the AAV2OVA vector, efficient antigen presentation on AAV8OVA vector transduction *in vivo* occurred within an early time period.

It has been thought that AAV vectors induce a low cellular immune response against the capsid because AAV vector transduction does not produce any endogenous capsid protein. However, clinical trials have suggested that capsid antigen can be cross-presented on AAV transduction and that AAV-transduced target cells can be eliminated by

FIG. 5. The kinetics of capsid antigen presentation after AAV8OVA transduction in mice. Particles of AAVOVA/AAT virus (1×10^{11}) were injected intravenously into C57BL/6 mice, and at the indicated time points 5×10^6 CFSE-labeled OT-1 T cells were transferred. Ten days after transfer proliferation of CD8⁺ OT-1 T cells was measured by flow cytometry. **(A)** Representative flow cytometric histograms. **(B)** Average T cell proliferation and standard deviation for four mice. **(C)** Average proliferation index (PI) and standard deviation. ** $p < 0.01$, * $p < 0.05$ compared with control mice without AAV treatment.

capsid-specific CTLs (Manno *et al.*, 2006; Nathwani *et al.*, 2011). In one clinical trial, an AAV2 vector was used to deliver F9 into the liver via the arterial route. High F9 expression was observed at week 4 after AAV2 injection and remained stable for 2 weeks before returning to the pre-gene therapy baseline. It was suggested that memory CTLs against the vector capsid were activated to clear AAV2-transduced hepatocytes (Manno *et al.*, 2006). Although capsid-specific CTL clones were isolated from the patient, and numerous experiments confirmed that capsid cross-presentation can also be induced on AAV vector transduction in animal models, the results from this study and others about the kinetics of AAV capsid cross-presentation, in which efficient antigen presentation occurs in earlier periods after AAV transduction, do not support the conclusion from the clinical trial (H. Li *et al.*, 2011). Alternative hypotheses have been proposed, such as cryptic epitopes derived from the F9 cDNA alternative reading frame (Li *et al.*, 2009a), which may provide an alternative rationale for the time course of F9 decline observed in patients.

AAV8 is one of the most efficient liver-transducing serotypes in animal models (Gao *et al.*, 2006; Wu *et al.*, 2008; C. Li *et al.*, 2011; Wang *et al.*, 2012). AAV8 has been employed to treat patients with severe hemophilia B in a phase 1 clinical trial (Nathwani *et al.*, 2011). Eight patients were enrolled for treatment with self-complementary AAV8 vectors encoding a codon-optimized human F9 transgene via peripheral vein

administration. At initial report, four of six patients have discontinued F9 prophylaxis without spontaneous bleeding after a follow-up of 6–16 months. One of two patients receiving a high dose of 2×10^{12} AAV8 particles per kilogram had elevated liver transaminases with a drop in F9 level on day 62 after gene transfer. Enzyme-linked immunosorbent analysis detected a capsid-specific T cell response at 9 weeks, suggesting that the decrease in F9 was related to capsid-specific CTL reactivity (Nathwani *et al.*, 2011). In this study we have demonstrated that the efficiency of antigen cross-presentation on AAV8 transduction *in vivo* was confined to the earlier time points, similar to that with the AAV2 vector. So, similarly, these results do not support the current interpretation from AAV8/F9 clinical trials. To completely validate *in vivo* mouse studies further experiments are needed to test whether the kinetics with human hepatocytes is different from that with mouse cells. In addition, alternative interpretations of AAV liver transgene expression and CTL response correlation remain open-ended and may require further clinical studies specifically designed to answer these concerns.

Several studies have demonstrated that muscular injection of AAV2 elicits a CTL response against capsid (Chen *et al.*, 2006; Li *et al.*, 2007; Wang *et al.*, 2007); however, the application of AAV8 vector did not induce a capsid-specific CTL response in mice (Wang *et al.*, 2007). Consistent with this observation, activation of capsid-specific T cells was not induced after muscular injection of AAV8OVA vector in this

study. One possibility is that AAV8 binding on the surface of APCs is not mediated by heparan sulfate proteoglycan, the primary receptor for AAV2 (Summerford and Samulski, 1998; Vandenberghe *et al.*, 2006). It has been demonstrated that heparan binding directs activation of T cells against AAV 2 capsid (Vandenberghe *et al.*, 2006). The lack of heparan binding by AAV8 may therefore account for the lack of T cell activation by this vector.

Results from the aforementioned two clinical trials in patients with hemophilia B suggest that capsid-specific CTL responses eliminate AAV-transduced cells at increasing vector doses (Manno *et al.*, 2006; Nathwani *et al.*, 2011). The data from our study are consistent with the finding that higher doses of vector result in elevated antigen presentation on the surface of target cells. To achieve optimal transgene expression and decrease antigen load from input AAV vectors, several approaches have been exploited: (1) generation of double-stranded AAV vectors that can induce more rapid and increased transgene expression compared with conventional single-stranded cassettes (McCarty *et al.*, 2001, 2003; Wu *et al.*, 2008); (2) modification of transgene cassette to enhance transgene expression through optimization of the promoter, poly(A), and/or transgene cDNA codon (Wu *et al.*, 2008); and (3) removal of empty particles in AAV preparations to decrease virus antigen load and increase transduction by decreasing competition for AAV binding and intracellular trafficking with full virions (Urabe *et al.*, 2006).

In conclusion, our study has demonstrated that AAV8 engineered with an OVA peptide can induce proliferation of OVA-specific T cells through transfection of plasmid and infection with virus, thus allowing for the monitoring of *in vivo* capsid antigen presentation kinetics. There is a dose dependency for AAV capsid antigen presentation *in vivo*. Last, the kinetics of capsid antigen cross-presentation from AAV8 is similar to that of AAV2 after *in vivo* administration—antigen presentation for both serotypes occurs within an early time-frame. Continued studies on the kinetics of capsid antigen presentation from AAV transduction *in vivo* will help in the design of effective approaches to prevent and block capsid-specific CTLs against AAV-transduced target cells in future clinical trials.

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Author Disclosure Statement

No competing financial interest exists.

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Address correspondence to:

Dr. Chengwen Li

or

Dr. R. Jude Samulski

Gene Therapy Center

7119 Thurston-Bowles, CB 7352

University of North Carolina at Chapel Hill

Chapel Hill, NC 27599

E-mail: chengwen@med.unc.edu; rjs@med.unc.edu

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