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Adeno-Associated Virus Capsid Structure Drives CD4-Dependent CD8⁺ T Cell Response to Vector Encoded Proteins

Lauren E. Mays,* Luk H. Vandenberghe,* Ru Xiao,* Peter Bell,* Hyun-Joo Nam,† Mavis Agbandje-McKenna,† and James M. Wilson2*

The immunological sequelae of adeno-associated virus (AAV)-mediated gene transfer in vivo is quite complex. In murine models, most AAV capsids are associated with minimal or dysfunctional T cell responses to antigenic transgene products. In this study we compared T cell activation against AAV2/8 and AAV2/rh32.33 vectors expressing nuclear-targeted LacZ (nLacZ), GFP, or firefly luciferase in murine skeletal muscle. We show that, unlike AAV8, AAVrh32.33 yields qualitatively and quantitatively robust T cell responses to both the capsid and transgene product. AAV2/rh32.33.CB.nLacZ, but not AAV2/8, drives a high degree of cellular infiltration and a loss of detectable transgene expression in C57BL/6 mice. However, cellular immunity to AAVrh32.33 is ablated in the absence of CD4, CD40L, or CD28, permitting stable β-galactosidase expression. Treatment of CD40L⁻/⁻ mice with the CD40 agonist, FGK45, failed to restore the CD8 response to AAV2/rh32.33.nLacZ, suggesting that additional factors are involved. Our results suggest that specific domains within the AAVrh32.33 capsid augment the adaptive response to both capsid and transgene Ags in a CD4-dependent pathway involving CD40L signaling and CD28 costimulation. Structural comparison of the AAV8 and rh32.33 capsids has identified key differences that may drive differential immunity by affecting tropism, Ag presentation or the activation of innate immunity. This murine model of AAV-mediated cytotoxicity allows us to delineate the mechanism of viral immune activation, which is relevant to the translation of AAV technology in higher order species. The Journal of Immunology, 2009, 182: 6051–6060.

Gene therapy holds great potential for the treatment of monogenic disease (1). However, the safety and efficacy of gene transfer can be negatively affected by host immune responses to either the delivery vehicle or the encoded protein (2). Adeno-associated virus (AAV)3 is a promising gene delivery vector due to its ability to achieve sustained, high-level expression of a packaged gene within target tissues (3, 4). AAVs are nonpathogenic and capable of transducing both dividing and nondividing cells. They belong to the genus Dependovirus, requiring a helper virus for productive infection (5). In fact, the first AAV serotypes were discovered as contaminants of adenoviral preps (6, 7). Since that time, numerous serotypes and over 120 capsid variants composing six phylogenetic clades and two clonal isolates have been described (8–14). Although the majority of capsid sequences are highly conserved (~80% homology to AAV2) (11), a divergent group of variants, as represented by AAV4, maintain only ~60% homology to AAV2, the prototype serotype (13). AAVrh32.33, a novel engineered vector isolated from rhesus macaques and phylogenetically closest to AAV4, is evolutionarily and structurally divergent from other AAVs. Importantly, its seroprevalence in human populations is significantly reduced compared with AAV2, AAV7, and AAV8 making it attractive for broad potential applications (15).

As a member of the Parvoviridae family, AAV is characterized by a small, 4.7-kb, ssDNA genome housed in a nonenveloped, icosahehedral capsid (16, 17). The genome consists of a nonstructural rep gene, and a structural cap gene, flanked by two inverted terminal repeats. The cap gene encodes three overlapping viral structural proteins, VP1, VP2, and VP3, expressed in a ratio of ~1:1:10 via alternative splicing and unconventional start codon usage (18). VP3 monomers compose ~90% of capsid quaternary structure and consist of a highly conserved eight-stranded β-barrel motif (ββ-β1) (19, 20). Due to this conservation, capsid architecture is maintained for all structurally mapped AAVs, regardless of primary sequence homology (9, 19–22). The majority of sequence variation falls within the surface loops linking these β-strands. Surface loops compose three protrusions surrounding a depression at the 3-fold axis of symmetry and dictate the unique phenotypes of each capsid (23–25). Evaluation of capsid biology using cross-packaged vectors confirms that capsid structure can affect tissue tropism, antigenicity, transduction efficiency, and vector performance (26, 27).

Due to the stability of expression of foreign transgene products in numerous murine tissues, AAV was generally considered minimally immunogenic for many years (3, 4, 28). However, recent
reports have demonstrated that it is possible to generate humoral or cell-mediated immune responses to vector encoded proteins (29–33). Remarkably, even in cases in which stable expression of a foreign transgene product is achieved in murine models, expression of the identical transgene product in non-human primates is typically transient, accompanied by a substantial IFN-γ-producing T cell response and a brief elevation in liver transaminases (G. Gao, Q. Wang, R. Calcedo, L. Mays, P. Bell, L. Wang, R. Grant, J. Sammiguel, B. Furth, and J. Wilson, manuscript in preparation).

In this study, we have modeled differential immune activation to AAV by identifying two capsid variants with distinct immune activation profiles in murine skeletal muscle: AAV8 and AAVrh32.33. Our data indicate that the structure of the AAV capsid can impact the threshold required to activate a CTL response to AAV, where certain capsid variants more readily activate cellular immunity. We show that, unlike AAV8, the AAVrh32.33 capsid drives qualitatively and quantitatively robust IFN-γ-producing T cell responses to both capsid and transgene Ags, which was consistent for all transgene products tested as well as in multiple strains of mice. Using this model, we aim to delineate mechanistic aspects of cellular immune activation to AAV vectors in mice, highlighting the role of CD4+ T cell help and costimulation.

Materials and Methods

Animals

Male C57BL/6, BALB/c, CD40L−/−, and CD28−/− mice (6–8 wk-old) were purchased from The Jackson Laboratory. CD40L−/− and CD28-deficient mice were on a C57BL/6 background. Mice were maintained in the Animal Facility of Translational Research Laboratories. All experimental procedures involving the use of mice were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Vector production and purification

Recombinant AAV vectors with viral capsids from AAV8 or AAVrh32.33 expressing nuclear-targeted LacZ (nLacZ), GFP, or luciferase were manufactured as previously described (28) by PennVector at the University of Pennsylvania (Philadelphia, PA). Briefly, an AAV cts-plasmid containing transgene cDNA driven by the chicken β-actin promoter and flanked by AAV2 inverted terminal repeats was packaged by triple transfection of human embryonic kidney 293 cells using an adenovirus helper plasmid (pAdΔ6F), and a chimeric packaging construct containing the AAV2 rep gene and the AAV8 or AAVrh3.33 cap gene. Vectors were purified by three rounds of cesium chloride gradient centrifugation. The genome titers (genome copies/GC per milliliter) of AAV vectors was determined by real-time PCR.

Animal treatments

Mice were anesthetized i.p. with ketamine (70 mg/kg of body weight) and xylazine (7 mg/kg of body weight). A total of 1011 GC of recombinant AAV vector was injected into the anterior tibialis muscle in a volume of 50 μl. Mice were treated i.p. with 0.25 mg of MR-1 on days 0, 2, and 4 relative to vector injection. In preliminary studies using this treatment protocol >99% of CD4+ T cells were depleted. To block CD40L in mice i.p. with 0.1 mg of GK1.5 on days −2, 0, 2, and 4 relative to vector injection. In order to study these treatments we used an imaging system. The 150 mg/kg of D-luciferin substrate was i.p administered animals (ketamine (35 mg/kg) and xylazine (5 mg/kg), i.p.). At various time points, vector postinjection mice were sacrificed by CO2 inhalation followed by cervical dislocation to harvest spleen and muscle.

Real-time in vivo imaging

In vivo bioluminescent imaging was performed with the Xenogen IVIS imaging system. The 150 mg/kg of D-luciferin substrate was i.p. administered, and the luminescence was captured from ventral views. Mice received D-luciferin (Caliper) exactly 15 min before imaging and were anesthetized during this interval (10 min before imaging). Anesthetized mice were then placed in the IVIS Lumina Imaging System and imaged (exposed for 10–30 s). Two mice were imaged at each time. Regions of interest from displayed images were drawn on the sites of vector injection (left hind limb) and were quantified as total flux (photons per second) being released by luciferase activity using Living Image 2.5 software (Caliper-Xenogen).

Histology and transgene detection

To examine expression of nuclear β-galactosidase (β-gal), X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopuranoide) staining on cryosections from snap frozen muscles was performed according to standard protocols (34). Sections were slightly counterstained with Fast Red to visualize nuclei. Muscles expressing GFP were fixed overnight in formalin, washed in PBS for several hours, and then snap frozen for sectioning. Cryosections were mounted in Vectashield containing DAPI (4’,6-diamidino-2-phenylindole; Vector Laboratories) to show nuclei.

Immunostaining

To analyze the cell types within infiltrates, immunostaining and confocal microscopy was performed. Frozen sections were fixed in acetone at −20°C for 5 min, blocked in PBS containing 1% donkey serum, and incubated with rat Abs against CD8 (1/20; BD Pharmingen) followed by TRITC-labeled donkey anti-rat Abs (Jackson ImmunoResearch Laboratories) and rabbit Abs against Foxp3 (1/20; BioLegend). Sections were then stained with Cy5-labeled donkey anti-goat and FITC-labeled donkey anti-rabbit Abs (all from Jackson ImmunoResearch Laboratories) and finally mounted in Vectashield with DAPI. All Abs were diluted in PBS with 1% donkey serum and slides were washed several times in PBS after each fixation and incubation step. Images were acquired with a Zeiss LSM 510 confocal microscope and pseudocolored using the Zeiss LSM Image Browser.

Morphometric analysis

To quantify GFP expression, images were taken of the area with the strongest GFP fluorescence in each muscle section with a 10X objective at identical settings. The brightness of each image was measured with ImageJ software (W. Rasband, National Institutes of Health, http://rsb.info.nih.gov/ij/) on a scale from 0 (darkest) to 255 (brightest) and averaged for each group. To measure nuclear β-gal expression, images of muscle sections showing the area of strongest expression were taken with a 4X objective and nuclei that stained positive with X-Gal were counted using AnalySIS (Soft Imaging System) and ImageJ software. A macro was generated under AnalySIS that performed the following operations sequentially: 1) convert image into gray scale, 2) perform shade correction (correct for uneven illumination caused by the microscope), 3) set threshold between 0 and 120 (selects the X-Gal-positive areas), 4) perform phase color-coding (shows the selected areas in the image), 5) binarize image, and 6) invert image. The resulting picture is a black and white image that shows the X-Gal-positive areas in black over a white background. This image was then again converted into gray scale and opened in ImageJ. Black dots with a minimum size of 15 pixels (corresponding to β-gal-positive nuclei) were counted with ImageJ and values averaged for each group.

MHC class I tetramer staining

PE-conjugated MHC class I H2-Kb-1CPMYARV tetramer complex was obtained from Beckman Coulter. At various time points after vector injection, tetramer staining was performed on heparinized whole blood cells isolated by retro-orbital bleeds. Cells were stained for 30 min at room temperature with PE-conjugated tetramer and FITC-conjugated anti-CD8ε (Ly-2) Ab (BD Pharmingen). RBC were then lysed and cells were fixed with iTag MHC tetramer lysis solution supplemented with fix solution (Beckman Coulter) for 15 min at room temperature. The cells were then washed three times in PBS and resuspended in 0.01% BD CytoFix (BD Biosciences). Data were gathered with an FC500 flow cytometer (Beckman Coulter) and were analyzed with FlowJo analysis software (Tree Star). In the analysis, lymphocytes were selected on the basis of forward and side scatter characteristics, followed by selection of CD8+ cells, and then the tetramer-positive CD8+ T cell population.
Intracellular cytokine staining

Splenocytes were plated at two densities (10^5 and 2.5 x 10^5 cells/ml) in triplicate on 96-well round-bottom plates. Cells were centrifuged for 5 min at 1600 rpm at room temperature, resuspended in fresh 1-L medium, and centrifuged again. The cell pellet was resuspended in T cell assay medium (DMEM, Cellgro; Mediatech), 10% heat-inactivated FBS (HyClone), 1% penicillin-streptomycin (Cellgro; Mediatech), 1% L-glutamine, 10 mM HEPES (Cellgro; Mediatech), 0.1 mM nonessential amino acids (In Vitro Pen Culture Media, Mediatech), and 0.05% Tween (Amersham Biosciences) gradient layer and centrifuged for 20 min at 2000 rpm at room temperature to remove RBC. The lymphocyte band was then recovered and further washed two times in PBS/1% FBS.

To determine the number of cells secreting IFN-γ in response to antigenic stimulation, an IFN-γ ELISPOT assay was performed according to the manufacturer’s instructions (BD Biosciences). Briefly, 96-well plates were coated with capture Ab overnight at 4°C, and blocked for 2 h at 25°C with RPMI 1640, 10% FBS, 1% penicillin-streptomycin-L-glutamine. Splenocytes were plated at two densities (10^5 and 2.5 x 10^5 cells/ml) in T cell assay medium supplemented with 1 μg/ml β-gal CD8 H2-K^K T cell epitope (ICPMYARV; Mimotopes), 2 μg/ml GFP or luciferase peptide libraries, or 2 μg/ml AAVm or AAVrh32.33 capsid peptide libraries were added to the corresponding experimental wells. Control cells were incubated without peptide, or in the presence of PMA (0.05 μg/ml) and ionomycin (1 μg/ml) (PMA/I; Sigma-Aldrich). Cells were incubated for 5 h at 37°C, in 10% CO2. Following the stimulation, cells were washed and stained with FITC-conjugated anti-mouse CD8α (Ly-2) Ab (BD Pharmingen) for 20 min at 4°C. Cells were washed with PBS/1% FBS, then permeabilized in Cytofix/Cytoperm solution at 4°C for 20 min. Cells were washed again in 1X Perm Wash Buffer and stained with anti-cytokine Abs including anti-mouse IFN-γ PE, TNF-α, PE-Cy7, and IL-2 allophycocyanin (BD Pharmingen) for 45 min at 4°C. Following incubation, cells were examined by flow cytometric analysis. Samples were acquired on an FC500 (Beckman Coulter) and analyzed using FlowJo software (Tree Star).

IFN-γ ELISPOT

Splenocytes from treated mice were harvested as described. Following isolation and washing steps, splenocytes were overlaid on a Ficol-Paque (Amersham Biosciences) gradient layer and centrifuged for 20 min at 2000 rpm at room temperature to remove RBC. The lymphocyte band was then recovered and further washed two times in PBS/1% FBS.

To determine the number of cells secreting IFN-γ in response to antigenic stimulation, an IFN-γ ELISPOT assay was performed according to the manufacturer’s instructions (BD Biosciences). Briefly, 96-well plates were coated with capture Ab overnight at 4°C, and blocked for 2 h at 25°C with RPMI 1640, 10% FBS, 1% penicillin-streptomycin-L-glutamine. Splenocytes were plated at two densities (10^5 and 2.5 x 10^5 cells/ml) in T cell assay medium supplemented with 1 μg/ml β-gal CD8α (Ly-2) Ab (BD Pharmingen) for 30 min at 4°C. Cells were washed with PBS/1% FBS, then permeabilized in Cytofix/Cytoperm solution at 4°C for 20 min. Cells were washed again in 1X Perm Wash Buffer and stained with anti-cytokine Abs including anti-mouse IFN-γ PE, TNF-α, PE-Cy7, and IL-2 allophycocyanin (BD Pharmingen) for 45 min at 4°C. Following incubation, cells were examined by flow cytometric analysis. Samples were acquired on an FC500 (Beckman Coulter) and analyzed using FlowJo software (Tree Star).

AAVrh32.33 model building

AAVrh32.33 shows highest sequence similarity to AAV4 among the AAV serotypes for which crystal structures are available. Thus the AAVrh32.33 capsid on vector performance and transgene stability in murine skeletal muscle. We compared AAV2/8 with AAVrh32.33 in vivo following an i.m. injection of 10^{11} GC in C57BL/6 and

**FIGURE 1.** Stability of transgene expression in mouse skeletal muscle. C57BL/6 male mice were injected in the left tibialis anterior with 10^{11} GC of AAV2/8, and AAV2/rh32.33 vectors expressing the classical reporter molecules nLacZ (A), GFP (B), and rLuc (C). At 7 and 63 days postinjection mice were sacrificed and skeletal muscle harvested for X-Gal histochemical stain (A) and immunohistochemistry (B). Live imaging was performed using a Xenogen imager to quantify luciferase expression in the skeletal muscle (C). Data are mean ± SD for four mice per group. Images represent regions of highest expression within an individual section. Statistical analysis compared AAV2/8 with AAV2/rh32.33 vectors for each time point. *p < 0.001, by unpaired Student’s t test.

**Results**

Statistical analysis was performed using the SigmaStat 3.5 program (SPSS). Statistical significance was set at p < 0.05 and statistical power at 0.80. Results are presented as the sample average ± SD. Two-group comparisons were made using the Mann-Whitney rank sum U test when data were not normally distributed, or the unpaired Student’s t test when data sets followed a normal distribution. ANOVA Student-Newman-Keuls test was used for multiple group comparisons.

Functional comparison between AAV8 and AAVrh32.33 vectors

Our initial objective was to characterize the effect of the AAV capsid on vector performance and transgene stability in murine skeletal muscle. We compared AAV2/8 with AAVrh32.33 in vivo following an i.m. injection of 10^{11} GC in C57BL/6 and
BALB/c mice. The onset and stability of expression was monitored using the classical reporter molecules nLacZ, enhanced GFP, and firefly luciferase (ffLuc). AAV2/8 was stable with all transgene products tested, but this result did not hold true for AAV2/rh32.33 (Fig. 1). AAV2/8.nLacZ expression was stable through day 63 with no visible signs of infiltration (Fig. 1A). AAV2/rh32.33.nLacZ showed a comparable degree of H9252-gal expression to AAV2/8 at day 7; however, by day 63 the number of H9252-gal-positive cells was substantially reduced. At day 63, few residual H9252-gal-positive fibers remained in the BALB/c strain, whereas expression in C57BL/6 mice was completely lost (Fig. 1A). There were no discernible differences in the onset of GFP expression from AAV2/8 and AAV2/rh32.33 by day 7 (Fig. 1B). In both strains, GFP expression from both AAV2/8 and AAV2/rh32.33 increased between days 7 and 63. The number of GFP-positive fibers at day 63 was slightly lower with AAV2/rh32.33 compared with AAV2/8. Luciferase expression was significantly higher with AAV2/8 vs AAV2/rh32.33 at all time points (Fig. 1C). Unlike the decline in transgene expression observed with AAV2/rh32.33.nLacZ and GFP, total flux of luminescence remained high for both AAV2/8.ffLuc and AAV2/rh32.33.ffLuc vectors through day 63.

The effect of recombinant AAV vector capsid on T cell responses toward capsid and transgene Ags

The decline in transgene expression observed with AAV2/rh32.33,nLacZ and GFP, but not AAV2/8 vectors, led us to investigate whether AAV2/rh32.33 generated a greater degree of cellular immunity toward the transgene product. At day 28, lymphocytes were isolated from whole blood and stained with a PE-conjugated H-2Kb-ICPMYARV tetramer together with a FITC-conjugated anti-CD8 Ab to determine the percentage of nLacZ-specific CD8+ T cells in the total CD8+ T cell population (Fig. 2B). Data are mean ± SD for four mice per group. Statistical analysis compared AAV2/8 with AAV2/rh32.33 at day 28, *p < 0.001, by Mann-Whitney rank sum U test (A) or unpaired Student’s t test (B).
638 SFU/10^6 splenocytes in BALB/c mice and 1264 SFU/10^6 splenocytes in their C57BL/6 counterparts (Fig. 2A). The same trend was observed with GFP in C57BL/6 mice, where AAV2/8 vectors generated little to no IFN-γ-producing GFP-specific T cells (66 SFU/10^6 splenocytes), whereas AAVrh32.33 drove a significantly greater response (495 SFU/10^6 splenocytes). In BALB/c mice in which there is a dominant CD8^+ T cell epitope to luciferase, a less dramatic difference in transgene response is observed between AAV2/8 and AAV2/rh32.33, with both generating high cell numbers at 463 and 545 SFU/10^6 splenocytes, respectively (Fig. 2A). This suggests that highly immunodominant transgenes may overcome the threshold required to generate T cells even in the presence of the AAV8 capsid. The T cell response to luciferase was also high for both vectors, in both mouse strains (Fig. 2A). Although it is possible for AAV2/8 vectors to elicit transgene T cell responses in some cases, under no circumstances was AAV2/8 able to generate T cell responses greater than AAV2/rh32.33.

Splenocytes were also stimulated with the AAV8 and AAVrh32.33 capsid peptide libraries to measure capsid-specific IFN-γ-producing T cell responses in C57BL/6 and BALB/c mice. Fig. 2B shows that AAV2/8 vectors generated little to no capsid-specific T cells in either strain (<25 and <50 SFU/10^6 splenocytes in BALB/c and C57BL/6 mice, respectively), consistent with previous reports. The AAVrh32.33 capsid, in contrast, was highly immunogenic irrespective of transgene, generating strong AAVrh32.33-specific IFN-γ-producing T cells in both BALB/c (150–200 SFU/10^6 splenocytes) and C57BL/6 mice (1000–1400 SFU/10^6 splenocytes). It is important to note that capsid T cell responses were not cross-reactive. For example, cells isolated from mice injected with AAV2/rh32.33.nLacZ did not respond when stimulated with the AAV8 capsid peptide library and vice versa (data not shown).

Characterization of the T cell response to AAV and AAVrh32.33

For a more in depth comparison of the T cell responses to these vectors, we focused on AAV2/8 and AAV2/rh32.33 expressing nLacZ in C57BL/6 mice, as it provided the most definitive example of differential immune activation and the influence of AAV capsid on the generation of immunity. An evaluation of the kinetics of this response confirmed that AAV2/8.nLacZ generates minimal T cell activation to either the AAV8 capsid (Fig. 3A) or to the nLacZ transgene, by IFN-γ ELISPOT (Fig. 3B) or nLacZ-specific MHC class I tetramer stain (Fig. 3B). When compared with mice injected with sterile PBS alone, the T cell responses to AAV2/8.nLacZ capsid do not rise above background levels at any point during the response (Fig. 3A and data not shown). In contrast, T cells to the AAVrh32.33 capsid are barely above background at day 7, expand and peak at around 4 wk postinjection, then contract over time through day 63 (Fig. 3A). A similar kinetic response to the nLacZ transgene was observed from the AAV2/rh32.33 vector (Fig. 3B).

The kinetics of the T cell response to AAV2/rh32.33 and AAV2/8 were consistent with the degree of cellular infiltration present in the muscles of these mice 28 days postinjection with
vector (Fig. 4). At the peak of the response, mice injected with AAV2/rh32.33 had a large number of cellular infiltrates detectable by X-Gal histochemical stain (Fig. 4A). The degree of cellular infiltration to AAV2/8.nLacZ was minimal, reflecting what is seen when mice are injected with PBS alone (Fig. 4A and data not shown). The difference in total cellular infiltration was confirmed by confocal microscopy (Fig. 4B), where AAV8 shows little infiltration of CD4+ or CD8+ T cells. In contrast, these cells are highly abundant in mice injected with AAV2/rh32.33.nLacZ (Fig. 4C). Sections were stained for Foxp3, a marker for regulatory T cells; very few Foxp3+ CD4+ T cells were observed (Fig. 4B).

At 3 wk postinjection of AAV2/8 or AAV2/rh32.33 vectors, splenocytes were harvested and stained for expression of IFN-γ, TNF-α, and IL-2 by CD8+ T cells using intracellular cytokine staining. Before staining, cells were stimulated with either the AAV8 or AAVrh32.33 capsid peptide libraries (Fig. 5A) or the β-gal CD8-dominated epitope ICPMYARV (Fig. 5B) to determine the capsid- and transgene-specific T cell responses. The AAV8 capsid library was used to stimulate cells from AAV2/8-injected animals and the AAVrh32.33 capsid library was used to stimulate AAV2/rh32.33-injected animals. Data are expressed as the percentage of CD8+ T cells capable of secreting individual or multiple cytokines. CD8+ T cells to AAV2/rh32.33.nLacZ capsid were highly functional; substantial IFN-γ, TNF-α—producing single positive and double positive populations were observed. Single, double, and triple positive populations involving IL-2 production were present, although very few cells were able to express IL-2. The same trends were observed when the cells from these animals were stimulated with the β-gal CD8-dominated epitope (Fig. 5B). Cytokine production by CD8+ T cells from mice injected with AAV2/8.nLacZ was low following stimulation with the AAV8 capsid library or β-gal-dominant peptide, reflecting the levels seen in mice injected with a PBS vehicle control (Fig. 5 and data not shown).

The role of CD4+ Th cells and costimulation in generating capsid and transgene responses

One potential mechanism of T cell activation to AAV2/rh32.33.nLacZ involves CD4+ T cells providing help to prime a functional CD8+ response. To test this hypothesis C57BL/6 mice were injected with AAV2/rh32.33.nLacZ in the presence of the CD4-depleting Ab, GK1.5. The peak IFN-γ-producing T cell response to capsid and transgene was monitored by ELISPOT. The nLacZ-specific CD8+ T cell response was evaluated by MHC class I tetramer stain, and muscles were sectioned to analyze cellular infiltration and expression stability by X-Gal histochemical stain (Fig. 6). Intracellular cytokine staining was also performed to characterize the functionality of the CD8+ T cell responders (Fig. 5). In the absence of CD4+ T cell help, capsid- and transgene-specific T cell responses were completely ablated (Fig. 6). The remaining CD8+ T cell population was no longer able to produce IFN-γ, TNF-α, or IL-2 in any combination above background levels (Fig. 5). In the absence of a T cell response, β-gal expression in the skeletal muscle was stable at day 28 with no visible signs of cellular infiltration, in contrast to what is normally seen following AAV2/rh32.33.nLacZ (Fig. 6).

One proposed mechanism of CD4+ T cell help involves CD40L (CD154) on the CD4+ T cell licensing the APC by signaling through CD40. The “licensed” APC then up-regulates expression of proinflammatory cytokines, molecules involved in Ag processing and presentation, as well as cell surface molecules including B7 (CD80/86). B7 is a costimulatory molecule that then interacts with CD28 on the CD8+ T cell to provide a necessary second signal during T cell priming. We examined whether blockade of
FIGURE 7. Structural comparison of the AAV8 and AAVrh32.33 capsids. A. Superimposition of the VP1 monomer of AAV8 (green) and the predicted model for AAVrh32.33 (orange). The major differences between AAV8 and AAVrh32.33 are located in variable regions I and III-VII. B. Predicted AAVrh32.33 VP3 structure (orange monomer) and the icosahedral 5-, 3-, (3f), and 2-fold (2f) symmetry VP monomers (all in light gray) inside gray surface density. Variable loops of AAVrh32.33 that differ from AAV8 appear as colored regions on the capsid surface: I-red, II-dark green, III-yellow, IV-green, V-cyan, VI-blue, VII-magenta, VIII-purple, IX-dark gray. The triangle defines a viral asymmetric unit bounded by 5-, 3-, and 2-fold icosahedral symmetry axes.

Discussion

Understanding the immune consequences of AAV gene therapy is pivotal in the translation of this vector platform to the clinic. Vectors based on AAV fail to elicit cellular immunity to antigenic transgene products and give rise to long-term transgene expression in most murine models, which is a desirable property for gene therapy (2). Recent studies have better characterized the nature of the T cell responses to vectors based on a number of commonly used AAV capsids showing the production of dysfunctional transgene-specific T cells (40, 41), which may reflect the induction of tolerance (42). However, AAV-mediated expression of a foreign transgene in non-human primates is transient in nature, as the transgenic Ag raises a destructive cellular immune response (Gao et al., manuscript in preparation). In addition, the generation of CDS T cells to the AAV capsid in a clinical trial of liver-directed gene transfer, and the concurrent development of liver toxicity, is another illustration of the limitations of our current animal models (32).

A better understanding of the critical vector-cell interactions that drive or suppress effector T cell responses would be helpful in predicting and managing vector-host interactions in the clinic. One approach we have taken is to use natural variation in AAV capsid structure to map structural domains that may mediate these key steps in T cell activation. To this end, we identified two structurally related AAV capsids from natural variants AAV2/8 and AAV2/rh32.33 that produce qualitatively different effector T cell responses to the transgene product. The goal of this study was to better understand the key cellular steps in CDS T cell activation where these two vectors diverge.

By comparing AAV2/8 to AAV2/rh32.33 vectors expressing nLacZ in murine muscle, we were able to model differential immune activation to the vector capsid and vector encoded transgene. AAV8.nLacZ elicits virtually no immune response to either nLacZ or the capsid, consistent with previous reports of aberrant T cell responses in mice (40, 41, 43). AAVrh32.33.nLacZ, however, overcomes the threshold required for immune activation, to generate a strong T cell response to both the AAVrh32.33 capsid as well as the nLacZ transgene. T cell responses correlate with a high degree of cellular infiltration in the skeletal muscle and a loss of β-gal expression over time, reflecting what is normally seen with AAVrh32.33 differs from both AAV2 and AAV8 capsids by over 32%. A superimposition of viral protein (VP) monomers of the AAVrh32.33 model and the AAV8 crystal structure shows that the core β-barrel is highly conserved as has been observed among all the known parvovirus structures. Structurally variable loops lie interspersed between the β-strands (Fig. 7A). Structural differences between the AAV8 and AAVrh32.33 capsids were identified in each of the nine variable regions (I to IX) defined when comparing AAV2 and AAV4 (25). This result is consistent with the fact that AAV8 is highly homologous to AAV2, and AAVrh32.33 is similar to AAV4, respectively. The most prominent differences, due to insertions or deletions of amino acids residues in AAVrh32.33 relative to AAV8, were observed in variable regions I and III-VII (Fig. 7A). These variable regions are spread throughout the VP monomer, but are clustered from symmetry related VP monomers on the capsid surface when displayed in the context of a viral asymmetric unit in a surface representation of nine VP monomers (Fig. 7B). For instance in Fig. 7B, regions IV (green) and VIII (purple) of a monomer are adjacent to regions V (cyan) and VI (blue) of a 3-fold-related monomer. The most variable regions, I (red), III (yellow), IV (green), V (cyan), VI (blue), and VII (magenta), are clustered around the icosahedral 2- and 3-fold symmetry axes.

Structural differences between the capsids of AAV8 and AAVrh32.33

Phylogenetically, AAV8 and AAVrh32.33 are two of the most divergent capsid variants. In comparison, the AAV2 capsid differs from AAV8 by 16.5% of VP1 amino acid composition, whereas...
a more immunogenic vector, such as adenovirus-expressing LacZ (44). Our hypothesis would predict that capsid variation impacts on T cell activation through interactions with the host cell such as antigenicity, receptor binding, and transduction efficiency (24, 25).

The ability of AAVrh32.33 to drive capsid and transgene T cell responses that are equal to or greater than responses seen with AAV8 was consistent for nLacZ, GFP, and IIluc in both C57BL/6 and BALB/c mice. Otherwise stated, the effect of capsid structure on the CTL response was independent of transgene product or MHC haplotype. Consistent with what others have shown, the magnitude of the transgene-specific T cell response to these vectors was influenced not only by the capsid, but also by the inherent antigenicity of the encoded transgene (45). For instance, in C57BL/6 mice, where GFP is minimally immunogenic, AAV8 was unable to generate GFP-specific T cells (46). However, in BALB/c mice where there is a strong immunodominant epitope to GFP, AAV8 elicited a substantial GFP-specific response, suggesting that the immunogenicity of the transgene overcame the threshold for generating T cells and bypassed the need for support from the AAV capsid (47). The AAV2/rh32.33 capsid was able to overcome this threshold and elicit a strong GFP-specific T cell response even in the C57BL/6 strain. It is also important to note that the presence of transgene-specific T cells did not always correlate with a loss of transgene expressing cells over time. Although AAV2/rh32.33.nLacZ and GFP resulted in a decline in transgene expression by day 63, expression from AAV2/rh32.33.11luc was stable despite a large number of CD8\(^+\) T cells to the luciferase transgene. The presence of substantial numbers of dysfunctional transgene-specific T cells at the peak of the response may be the result of persistent, high level Ag expression leading to T cell exhaustion and the loss of functionality (48).

The ability of capsid structure to influence not only the capsid-specific but also the transgene-specific CD8\(^+\) T cell response could be interpreted in two different ways: either the AAVrh32.33 capsid is augmenting a normally weak cellular response to AAV, or the AAV8 capsid is suppressing the standard CTL response seen with AAVrh32.33, or some combination of the two ideas. Several serotypes, such as AAV2, AAV7, and AAV8, are known to generate dysfunctional CD8\(^+\) T cell responses to an HIVgag transgene in mice (40, 41). Although HIVgag-specific CD8\(^+\) T cell responses are primed by these vectors, these cells are unable to secrete cytokines or undergo proliferation in response to Ag re-exposure. Moreover, in the current study, AAV8.nLacZ was unable to generate a substantial CD8\(^+\) T cell population to either capsid or transgene Ags. The aberrant T cell responses to AAV8 capsid-based vectors could result from anergy, functional exhaustion, or active suppression. Cao et al. (42) has provided evidence that hepatic gene transfer of AAV2 vectors results in the induction of CD4\(^+\)CD25\(^+\)Foxp3\(^+\) regulatory T cells. The authors suggest that regulatory T cells are capable of inducing tolerance, suppressing transgene-specific immune responses. Lin et al. (41), also noted an increase in CD4\(^+\)CD25\(^+\) T cells following AAV2/HTLVHIVgag immunization; however, the depletion of CD25\(^+\) regulatory T cells did not improve the proliferative capacity of the transgene-specific CD8\(^+\) T cells in this case. Analysis of CD4 T cell responses to capsid and transgene Ags following AAV2 gene transfer to mice and nonhuman primates showed poor proliferative responses and little to no secretion of cytokines (49). The classic AAV serotype, AAV2, has been shown to elicit poor innate immune activation when compared with adenovirus, potentially explaining the weak activation of CD4 T cells (50). Delayed or incomplete activation of CD4\(^+\) T cells by AAV could prevent proper APC recruitment, licensing, and the expression of costimulatory molecules required to avoid functional anergy (45). However, studies attempting to restore the proliferative capacity of AAV7-induced HIVgag-specific CD8\(^+\) T cells by coadministration of adjuvants suggested that TLR-induced nonspecific inflammation alone failed to generate CTLs to AAV (41). In this same study, the authors demonstrate that AAV-induced transgene-specific T cells express markers of exhaustion such as PD-1; however, blockade of PD-1 is unable to rescue the proliferative capacity of these cells (41). Ultimately, the affect of capsid structure on cellular immunity is multifaceted and both AAV8-mediated suppression as well as AAVrh32.33-mediated enhancement may play a role. Future studies evaluating the role of AAV capsid structure in specific vector-host cell interactions are necessary to further delineate this mechanism.

Our next goal was to use this model of cellular immunity to characterize the mechanism of T cell activation to AAVrh32.33 in murine skeletal muscle. Sarukhan et al. (45) has demonstrated that AAV2 results in delayed CD4\(^+\) T cell kinetics when compared with the more immunogenic adenoviral vectors. The high degree of CD4 infiltration in skeletal muscle with AAVrh32.33, but not with AAV8, supports the hypothesis that CD4 T cells are necessary to promote a CD8\(^+\) T cell response. Indeed, the depletion of CD4\(^+\) T cells in this system completely ablates the generation of CD8\(^+\) T cells to AAVrh32.33, concurrently alleviating the associated cytotoxic sequelae. Numerous studies have demonstrated the requirement for CD4 help in priming CD8\(^+\) T cell responses. However, certain pathogens, including acute lymphocytic choriomeningitis virus and Listeria monocytogenes, are CD4-independent (51–54). Using L. monocytogenes as a model, Shedlock et al. (51) has shown that CD8\(^+\) T cells responses are likely to require CD4 help when the pathogen is not immunogenic enough to directly activate APCs. Thus, the dependence of AAV on CD4\(^+\) T cell help is not surprising given the noninflammatory nature of the vector (50).

One proposed mechanism of CD4\(^+\) T cell help involves CD40L\(^+\) CD4\(^+\) T cells “licensing” CD40-expressing APCs that are then capable of priming naïve CD8\(^+\) T cells in a CD28-dependent fashion (55–57). Licensed APCs up-regulate the expression of costimulatory molecules (CD80/86), cytokines (namely, IL-12), and Ag processing and presentation machinery (58–60). Alternatively, CD40L-bearing CD4\(^+\) T cells may provide help directly by engaging CD40-expressing CD8\(^+\) lymphocytes (61). In the case of AAVrh32.33, ablation of CD8\(^+\) T cell responses to both capsid and transgene Ags in the absence of CD40L confirms that CD40-CD40L signaling is necessary to the CD4-dependent CD8\(^+\) T cell response. CD40-CD40L signaling is not always required to facilitate CD4\(^+\) T cell help: Lu et al. (62) demonstrated that dendritic cells from CD40\(^{-/-}\) mice were equally capable of being licensed by CD4\(^+\) Th cells as their wild-type counterparts in vitro. In vivo, well-characterized viral models demonstrate that the requirement for CD40-CD40L costimulation is pathogen-dependent. Although CD40L deficiency does not affect the CD8\(^+\) T cell response to lymphocytic choriomeningitis virus or herpes simplex virus, the primary CD8\(^+\) T cell response to vesicular stomatitis virus is significantly impaired in the absence of CD40-CD40L signaling (63, 64).

Many studies using CD4\(^{-/-}\) or CD40L\(^{-/-}\) mice have shown that exogenous CD40 activation using the agonistic Ab FGK45 can bypass the need for CD4 help, restoring the CD8\(^+\) T cell response (55–57, 60). However, our data show that this bypass is not the case with AAVrh32.33, where the CD8\(^+\) T cell response was not restored in CD40L\(^{-/-}\) mice supplemented with FGK45. These results indicate that CD40-CD40L signaling is necessary but not sufficient to support the generation of CD8\(^+\) T cells to both capsid and transgene Ags. This result may suggest a role for CD40L signaling beyond the activation of APCs or CD8s alone, as well as...
a role for additional molecules in priming CD8 cells. One additional molecule required for the induction of cellular immunity to AAVrh32.33 is CD28; the engagement of CD80/86 on the APC with CD28-CD8 T cells is a necessary second signal in T cell priming, without which primed T cells are often functionally anergic and unable to proliferate and secrete IL-2 in response to subsequent Ag encounter (65).

The availability of high resolution structures of AAV8 and a close relative of AAVrh32.33 called AAV4 provided an opportunity to observe regions on the surface that may mediate differential receptor binding relevant to the divergent T cell responses. These two capsids retain a substantial amount of three-dimensional structural similarity, although differences were noted at the previously described variable regions. The most substantial structural differences were mapped to six regions (I and III-VI) spanning variable sequences. At least four of these regions colocalize within a single surface domain as visualized by the assembly of nine VP monomers. Current efforts are directed at using this structural data to guide the design of hybrids between AAV8 and AAVrh32.33. Studies comparing the performance of hybrids in the presence or absence of specific structural domains will be necessary to map the capsid domain responsible for differential T cell activation.

In summary, our findings demonstrate that the AAVrh32.33 capsid, unlike other traditionally studied AAV serotypes, generates robust polyfunctional CD8+ T cells to both capsid and transgene Ags. These T cells appear to be activated in a classical, CD4-dependent pathway involving CD40L signaling and CD28 costimulation. Further comparison of the AAVrh32.33 capsid to the structurally and functionally divergent AAV8 capsid in this murine model will allow us to assess differential vector-host cell interactions to understand the mechanism by which traditional AAV serotypes avoid the activation of cellular immunity.

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References


47. Stripecke, R., M. Carmen Villacres, D. Skelton, N. Satake, S. Halene, and 

43. Vandenberghe, L. H., L. Wang, S. Somanathan, Y. Zhi, J. Figueredo, R. Calcedo, 

41. Lin, S. W., S. E. Hensley, N. Tatsis, M. O. Lasaro, and H. C. Ertl. 2007. Re-


60. Yang, Y., and J. M. Wilson. 1996. CD40 ligand-dependent T cell activation: 

58. Schuurhuis, D. H., S. Laban, R. E. Toes, P. Ricciardi-Castagnoli, M. J. Kleijmeer, 


474 – 478. 

393: 480 – 483. 

60: 2126 –2132. 

110: 1132–1140. 


Skelton et al. (2001) reported the enhanced green fluorescent protein (eGFP) is minimally immunogenic in C57BL/6 mice. Gene Ther. 8: 1813–1814.


Wherry et al. (2000) found that CD4 \(^+\) T cells in the generation of CD8 \(^+\) T cell memory. Science 297: 2060–2063.


Edelmann et al. (2001) reviewed the role of CD28/CD80 – 86 and CD40/CD154 signaling in host defense to primary herpes simplex virus infection. J. Virol. 75: 612–621.