Elimination of contaminating cap genes in AAV vector virions reduces immune responses and improves transgene expression in a canine gene therapy model

Z Wang1,2, CL Halbert3, D Lee4, T Butts1, SJ Tapscott3,5, R Storb1,2 and AD Miller3,6

Animal and human gene therapy studies utilizing AAV vectors have shown that immune responses to AAV capsid proteins can severely limit transgene expression. The main source of capsid antigen is that associated with the AAV vectors, which can be reduced by stringent vector purification. A second source of AAV capsid proteins is that expressed from cap genes aberrantly packaged into AAV virions during vector production. This antigen source can be eliminated by the use of a cap gene that is too large to be incorporated into an AAV capsid, such as a cap gene containing a large intron (captron gene). Here, we investigated the effects of elimination of cap gene transfer and of vector purification by CsCl gradient centrifugation on AAV vector immunogenicity and expression following intramuscular injection in dogs. We found that both approaches reduced vector immunogenicity and that combining the two produced the lowest immune responses and highest transgene expression. This combined approach enabled the use of a relatively mild immunosuppressive regimen to promote robust micro-dystrophin gene expression in Duchenne muscular dystrophy-affected dogs. Our study shows the importance of minimizing AAV cap gene impurities and indicates that this improvement in AAV vector production may benefit human applications.

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Keywords: AAV6 vectors; immune responses to AAV vectors; immune modulation; Duchenne muscular dystrophy

INTRODUCTION
Viruses are capable of efficiently transferring and expressing their genomes in many mammalian cell types, making viral vectors attractive for delivering genes to treat a variety of genetic diseases. However, mammals have a highly comprehensive immune system evolved to protect against invading viruses, including those used as viral vectors. This makes host immune responses major challenges for successful viral vector-mediated gene therapy. Historically, adeno-associated viral (AAV) vectors have been considered safe and less immunogenic than other vectors, such as adenoviral vectors. However, accumulating data from animal models and human trials have shown that both innate and adaptive immune responses can be elicited against AAV capsid (Cap) proteins that compromise long-term therapeutic efficacy of gene therapy.1,2

There are two sources of AAV Cap proteins that can induce host immune responses: one is the Cap protein from the input AAV virions,3–5 and the other is the Cap protein expressed from cap genes aberrantly packaged into AAV vectors during vector production.3–5 Conflicting data on whether cap gene expression is involved in inducing host immune responses against vector-transduced cells have been reported. For example, studies from Li et al.6 indicated that only vector-transduced cells that expressed de novo synthesized AAV Cap proteins were eradicated by AAV Cap-specific cytotoxic T lymphocyte (CTL) reactions, while other studies indicated eradication of AAV-transduced target cells by Cap-specific CTL in the absence of de novo Cap protein synthesis.4,5,9 It is possible that the disagreement in detecting host immune responses against AAV vector or in the degree of host immune responses to AAV vectors are influenced by the amount of AAV cap gene expression because of the differences in AAV vector preparation methods used by the different groups. A recent study demonstrated that cap genes are packaged into and expressed from AAV vectors produced by the standard methods.7 The authors further demonstrated that introduction of a large intron into the cap gene (captron) made the resultant gene too large to be packaged into AAV virions, prevented intact cap gene transfer and expression and eliminated Cap protein expression in vector-transduced cultured cells.7

Here, we tested the hypothesis that administration of an AAV vector modified to avoid cap gene transfer and in vivo expression would result in reduced immune responses and increased therapeutic gene expression compared with that of a standard AAV vector, following intramuscular administration in randomly bred wild-type dogs. We further investigated the effect of vector purification by cesium chloride (CsCl) gradient centrifugation, which removes unwanted empty AAV capsids, on AAV vector immunogenicity and transgene expression. We found that either approach reduced vector immunogenicity and improved transgene expression. Moreover, the lowest host immune responses and highest transgene expression were seen when both approaches were combined. Further, in dogs affected with...
Duchenne muscular dystrophy (DMD), the combination of both approaches resulted in reduction of host immune responses and robust transgene expression with an immuno-suppressive regimen that was less intense than the previously used immuno-suppressive regimen developed for AAV vector administration to the dystrophic muscles. Our study emphasizes the importance of minimizing AAV cap gene impurities and suggests that this simple improvement in AAV vector production may benefit human gene therapy applications.

RESULTS

AAV vector production

An AAV vector encoding canine blood clotting factor IX (cFIX) was used to avoid an immune response against the vector product in dogs and to allow detection of the vector product in the muscle, where cFIX is not normally made. For vector production, we used a standard AAV6 cap gene (carried by the plasmid pCMVCap6), which can be packaged and expressed at low levels by AAV vectors,7 or the AAV6 captron gene (carried by the plasmid pCaptron6), which is too large to be packaged into AAV virions.7 (Figure 1a). AAV vectors were generated by transfection of human embryonic kidney 293 cells with vector and packaging plasmids, by harvest and purification of virions on heparin columns and with or without final purification on CsCl gradients to eliminate empty virions (Figure 1b).

AAV Cap proteins in the vector preparations were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Figure 2). The AAV6 viral Cap proteins VP1, VP2 and VP3 were the major proteins present in most of the vector preparations, with the exception of the captron6 vector preparations made without CsCl purification, which displayed single additional protein bands of different sizes that did not co-migrate with any of the Cap proteins. Such additional proteins are not detected by an antibody that recognizes AAV6 VP1, VP2, and VP3 in crude AAV vector preparations before heparin column or CsCl gradient purification (Figure 2 in Halbert et al.). Therefore, the additional proteins are unlikely to be variant Cap proteins, for example, proteins generated from the captron gene by alternative splicing. We conclude that these additional proteins likely derive from the 293 cells used to make the AAV vector and likely have not affected our results or conclusions (see Discussion). As expected, the vector preparations that were subjected to additional CsCl purification displayed much lower Cap protein levels than preparations made without CsCl purification, when the same numbers of vector genomes were analyzed, most likely due to the elimination of empty AAV capsids.

Host cellular immune responses to AAV vector preparations

To determine the effects of elimination of cap gene transfer and CsCl vector purification on host immune responses to AAV vectors, we performed intramuscular injections in four groups of two dogs each with the following AAV6 vector preparations carrying the cFIX gene: group A, standard AAV production with heparin column purification only (dogs H218 and H316); group B, standard AAV production with heparin column plus CsCl gradient purification (Figure 1b).

Figure 1. AAV6 Cap expression plasmids and vector production scheme. (a) AAV6 Cap expression cassettes are shown. The pCaptron6 plasmid differs from pCMVCap6 by the inclusion of a large intron from the human EIF2S1 gene, as previously described.7 The CMV promoter present in both plasmids is shown at left with the transcription start sites indicated by arrows. The SV40 polyadenylation signal at the right side of the expression cassettes is indicated by p(A). (b) Vector production scheme.
purification (dogs H468 and H507); group C, captron AAV production with heparin column purification only (dogs H208 and H411); and group D, captron AAV production with heparin and CsCl purification (dogs H221 and H252). Each dog received 5 × 10^11 vector genomes of the AAV6 vector preparation at each of the four distinct locations in one limb, and an additional injection of buffer at a control site in the same limb, and all sites were marked with sterile sutures. The same muscles in the contralateral limb served as untreated controls. After 4 weeks after the injections, a time point previously shown to exhibit the maximal immune cell infiltration,^1^ the injected sites were imaged by magnetic resonance imaging (MRI) to detect inflammation, and one of the injection sites was then biopsied accordingly for histological analysis.

Our previous study^12^ demonstrated that cellular immune responses to AAV in the muscle can be detected as hyper-intense regions on T2-weighted MRI images, as well as increases in T2 values. Although hyper-intense regions were observed in the muscles of the four dogs injected with AAV vectors produced by using the cap6 gene with or without CsCl purification (Figure 3, top panels), and to a reduced extent in the two dogs that received the captron AAV vector without CsCl purification (Figure 3, bottom left panels), no inflammation was observed in the two dogs given the captron AAV vector with CsCl purification (Figure 3, bottom right panels). Increases in the T2 values, which assess the intensity of inflammation, were detected only in dogs with detectable hyper-intense regions on T2-weighted MRI images (Figure 4a). No increases in T2 values were detected in the muscle regions injected with buffer only, compared with the untreated contralateral limb muscle (data not shown). In addition, three-dimensional (3D) T2-weighted images were acquired to measure the total volume of inflammation, which assesses the extent of inflammation, for each injection site (Figure 4b). The volume of each inflammatory region correlated with corresponding hyper-intense region on T2-weighted image (Figure 3) and the T2 values (Figure 4a). Importantly, no increase in T2 values or any area of inflammation was detected in either dog that received the CsCl-purified captron6 AAV vector (Figure 4).

Table 1 provides statistical comparisons of the T2 measurements of inflammation for the four groups of dogs. There is a statistically significant increase in the T2 values for all vector-treated dog muscles compared with the untreated control muscle (P < 0.0001 to 0.0013), with the exception of muscle injected with the Captron6 CsCl-purified vector, which shows no difference (P = 0.998). In addition, there is a reduced immune response to the CsCl-purified Captron6 vector compared with the CsCl-purified Cap6 vector (P = 0.016) and a reduced immune response to the CsCl-purified Captron6 vector compared with the non-CsCl-purified Captron6 vector (P = 0.011). These results indicate that both the use of Captron6 and the CsCl purification contribute to the lack of detectable immune response to the CsCl-purified Captron6 vector.

Muscle biopsies were performed following MRI for immunohistochemical analysis of transgene expression and for histological detection of cellular immune responses. Representative micrographs revealed cFIX expression in the muscles of all animals, with the highest expression observed in animals that received the CsCl-purified captron6 AAV vector (Figure 5a). Note that many of the cFIX-positive muscle fibers in the left panels appear smaller than those in the right panels. This is because of the high level of muscle destruction and the smaller sizes of the regenerating muscle fibers in the left panels compared with the right panels. However, one can still observe some normal size fibers in the left panels, for example, the cell just below the middle of the right side of the dog H208 panel of Figure 5a. Levels of cFIX expression were quantitated by the extent of cFIX expression in multiple sections (Figure 5c).

Representative hematoxylin and eosin–phloxine staining on consecutive sections of muscle biopsy specimens from dogs in each of the four groups are shown in Figure 5b. In contrast to fairly uniform infiltration observed in muscles injected with AAV vectors produced using the standard cap gene, the muscles from dogs injected with the CsCl-purified AAV captron6 vector displayed very little inflammation. The degree of inflammation was quantitated by the number of inflammatory regions and by the size of each region in the biopsy (Figure 5c). The histology results were consistent with the MRI results and demonstrated that the captron AAV vector production strategy was able to significantly reduce host cellular immune responses to the vector. Furthermore, vector transgene expression (Figures 5a and c) appeared to inversely correlate with the observed immune response (Figures 5b and c).

Host-neutralizing antibody responses to AAV vector preparations

We measured serum-neutralizing antibody titers against AAV6 vectors before and 4 weeks after intramuscular vector injection (Table 2). Neutralizing antibodies were not detected in any of the dogs before vector injection. After injection, the strongest response was against the AAV6 vectors made using the standard cap gene in the absence of CsCl purification. The remaining vector preparations elicited much lower neutralizing antibody responses, with the lowest response being observed for the vector made with the captron gene plus CsCl purification. We hypothesize that the greatly elevated antibody response to the vector made using the cap gene without CsCl purification, in comparison to that of the other vector preparations, is due to a synergistic effect of the

Figure 3. T2-weighted MRI images at week 4. Dog identifiers are shown at the top left of each panel, the cap gene used for production of the vectors is shown at the left and the vector purification details are shown at the top of the Figure. Arrows point at hyper-intense areas where increased T2 signals were detected.

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higher incoming Cap protein associated with the non-CsCl-purified vector and prolonged expression of the standard cap gene in the target cells, but additional studies would be required to substantiate this hypothesis.

AAV captron vector allows the use of reduced intensity immunosuppression for treatment of dogs with DMD. Our previous studies demonstrated that a combination of the immunosuppressive agents cyclosporine (CSP), a calcineurin inhibitor that interferes in the signaling pathway required for T-cell activation and proliferation, and mycophenolate mofetil (MMF), which interferes in T-cell proliferation by blocking nucleotide synthesis, was not sufficient in preventing immune responses to AAV Cap proteins following intramuscular injections of AAV vectors into the muscles of dogs affected with DMD. A more potent immunosuppression regimen using three drugs, including anti-thymocyte globulin (ATG) from day 0 to day 2 (1 mg kg⁻¹ day⁻¹), CSP (15 mg kg⁻¹) from days –2 to day 2 (1 mg kg⁻¹ day⁻¹), and MMF (10 mg kg⁻¹) from days 0 to 184, was needed to...

**Figure 4.** T2 values and the volume of inflammatory regions. (a) T2 values in the vector-injected areas that showed hyper-intense signals (gray bars) and in similar areas from the same muscles in the untreated contra-lateral limb (black bars) are shown (mean values ± s.d.). (b) The volumes of inflammation in the injected areas with abnormal T2 signals are shown (mean values per injection site ± s.d.).

**Table 1.** Statistical comparison of T2 values for untreated and vector-injected muscles

| Comparison                        | Mean difference | 95% CI         | Significant difference | Adjusted P-value |
|-----------------------------------|-----------------|----------------|------------------------|-----------------|
| Untreated vs Cap6 CsCl⁻ vector    | –25.2           | –35.3 to –15.1 | Yes                    | <0.0001         |
| Untreated vs Cap6 CsCl⁺ vector    | –16.4           | –26.5 to –6.3  | Yes                    | 0.0021          |
| Untreated vs Captron6 CsCl⁻ vector| –17.4           | –27.5 to –7.3  | Yes                    | 0.0013          |
| Untreated vs Captron6 CsCl⁺ vector| –0.9            | –11.0 to 9.2   | No                     | 0.998           |
| Cap6 CsCl⁻ vector vs Cap6 CsCl⁺ vector | 8.8            | –4.0 to 21.6   | No                     | 0.24            |
| Cap6 CsCl⁻ vector vs Captron6 CsCl⁻ vector | 7.8            | –5.0 to 20.6   | No                     | 0.34            |
| Cap6 CsCl⁺ vector vs Captron6 CsCl⁻ vector | 24.3           | 11.5–37.1      | Yes                    | 0.0006          |
| Cap6 CsCl⁺ vector vs Captron6 CsCl⁺ vector | –1.0           | 13.8 to 11.8   | No                     | 0.999           |
| Cap6 CsCl⁻ vector vs Captron6 CsCl⁻ vector | 15.5           | 2.7–28.3       | Yes                    | 0.016           |
| Captron6 CsCl⁻ vector vs Captron6 CsCl⁺ vector | 16.5           | 3.7–29.3       | Yes                    | 0.011           |

Abbreviations: CI, confidence interval; CsCl⁻, vector made without CsCl gradient purification; CsCl⁺, vector made with CsCl gradient purification. Statistical comparison of T2 values for untreated muscles (n = 8) and vector-injected muscles (n = 2 for each vector preparation) was performed by using analysis of variance with correction for multiple comparisons by the Tukey’s method (with α = 0.05 and 95% CI) to obtain adjusted P-values.
Figure 5. Canine FIX expression and inflammatory responses detected in muscle biopsy samples at week 4. (a) cFIX expression was detected by immunohistochemistry (green) and cell nuclei are stained with DAPI (4,6-diamidino-2-phenylindole; blue). (b) Hematoxylin and eosin–phloxine staining. (c) Overall cFIX expression was estimated based on multiple stained sections from each dog, and the degree of inflammation was estimated based on the numbers of inflammatory regions and the extent of each region in the biopsy sections. Representative results from one dog in each treatment group are shown in panels (a) and (b) with the dog identification numbers at the lower left of each panel. Bars = 100 μm.
prevent the immune responses and achieve efficient transgene expression in DMD dogs. Because the captron AAV production strategy in combination with CsCl purification significantly reduced host cellular immune responses to AAV vectors, we tested whether CSP and MMF without ATG would be sufficient to suppress inflammatory responses to an AAV6 captron vector in the dystrophic dog muscles. Two DMD affected dogs received $5 \times 10^{11}$ vector genomes per site of an AAV6 captron vector carrying a canine micro-dystrophin (c-µdys) gene at distinct locations in the muscles in one limb, whereas the same muscles in the contra-lateral limb were left untreated as a control. CSP and MMF were used as described above.11,18 Muscle biopsies were taken 4 weeks, 6 months (at the time of discontinuation of immunosuppression) and 7 months (1 month after discontinuation of immunosuppression) after vector administration, and extensive expression of c-µdys was detected in myofibers at all time points in both dystrophic dogs without obvious inflammation and with normalized muscle histology (Figure 6). Importantly, extensive c-µdys expression was detected 1 month after cessation of immunosuppression, 7 months after vector injection.

**DISCUSSION**

A major obstacle in using AAV vectors for in vivo gene delivery has been the development of host immune responses to viral Cap proteins and transgene products both in animal models and in humans.11 AAV Cap proteins are capable of inducing CTL responses through both classical major histocompatibility complex class I antigen presentation and cross-presentation pathways5–7,10,20 with two sources of AAV Cap proteins as antigens: delivered AAV virions and endogenously expressed Cap protein from DNA impurities.5–7 In immune-competent dogs, we previously reported that local intramuscular injection of AAV1, AAV2 or AAV6 vectors into the muscle of wild-type or DMD dogs induced robust T-cell-mediated immune responses against AAV Cap proteins that peaked at 4 weeks after vector injection and eliminated transgene expression by week 10.10,12 In the current study, by performing intramuscular injection in wild-type dogs, we demonstrate that elimination of cap gene impurities by using an oversized captron gene significantly reduced the magnitude of host cellular immune responses to the AAV vectors and improved expression of the cFIX transgene carried by the vector. We also demonstrate that vectors produced by combining CsCl gradient centrifugation for removal of unwanted empty AAV capsids with the captron production system generated an even lower immune response. Further, combined elimination of endogenous and empty AAV Cap protein resulted in robust transgene expression in DMD dogs given MMF and CSP but not ATG. ATG had been a required component of the current immunosuppressive regimen for AAV injection in our DMD dog model.11,17 Contamination of AAV preparations with the cap gene has been observed by several groups of investigators,5–7,19 while the involvement of de novo-expressed AAV Cap protein in the induction of host cellular immune responses remained to be addressed. By using dendritic cells either pulsed with AAV2 vectors or with endogenous AAV2 Cap expression, Li et al.8 found that AAV2 Cap protein-specific CTL could be induced. However, the CTL only eliminated cells with endogenous AAV2 Cap protein expression but did not eliminate AAV2-transduced cells, both in vitro and in a mouse model.12 In our study, immune responses were induced to a much lower extent against AAV captron vectors compared with AAV vectors produced using the standard method, indicating a contribution of endogenously expressed AAV Cap to

![Figure 6. Robust expression of c-µdys after AAV vector treatment in DMD-affected dogs immunosuppressed with cyclosporine and mycophenolate mofetil only.](image-url)

**Table 2. Appearance of neutralizing antibodies in blood following AAV6 vector exposure**

| Cap expression plasmid | CsCl purification | Dog | Neutralizing antibody titer |
|------------------------|------------------|-----|-----------------------------|
|                         |                  |     | Before vector exposure | 4 weeks after vector exposure |
| pCMVcap6                | No               | H218| $<10$                      | 12,800 |
|                         | No               | H316| $<10$                      | 25,600 |
|                         | Yes              | H411| $<10$                      | 3200  |
| pCMVcap6                | Yes              | H468| $<10$                      | 3200  |
|                         | Yes              | H507| $<10$                      | 3200  |
|                         | Yes              | H221| $<10$                      | 3200  |
|                         | Yes              | H252| $<10$                      | 1200  |

Abbreviation: AAV6, adeno-associated virus type 6. *The neutralizing antibody titer is defined as the reciprocal of the highest dilution of serum that inhibited AAV6 vector transduction by $>50\%$ compared with the untreated vector. All assays were performed twice with identical results except those for dog H252, which gave titers of 800 and 1600. The average of 1200 is reported.*
the host’s cellular immune response. In contrast, studies by Hauck et al. found undetectable residual cap expression, and the studies suggested that preformed incoming AAV Cap protein was the major source of antigen recognized by T cells. In line with this study, we observed the lowest degree of cellular immune responses when AAV vectors were further subjected to CsCl gradient centrifugation to reduce the amount of incoming empty AAV capsids. The data also agree with other studies showing dose-dependent immune responses. Those studies demonstrate that input AAV Cap protein is another source of antigen responsible for induction of host CTL responses, through cross-presentation pathways, and indicate that reduction of AAV vector particle numbers are advantageous. Our studies support the involvement of both sources of AAV Cap protein in induction of host immune responses, including the incoming Cap proteins and Cap proteins expressed from aberrantly packaged cap gene DNA.

Our AAV vector preparations were largely free of proteins other than the AAV Cap proteins, with the exception of two AAV captron vector preparations made without CsCl purification, which displayed single protein bands of different sizes that did not co-migrate with (Figure 2) and appear unrelated to (Figure 2, Halbert et al.) any of the Cap proteins. Because we previously had not observed such additional bands, we did not perform the gel electrophoresis analysis before use of the vectors in dogs but performed the analysis for confirmation after the animal studies were underway. Additional studies showed that if detergent is left out of the salt solution used to wash heparin columns before AAV vector elution, both of these additional proteins can be found in the eluted vector preparations. In summary, the additional proteins found in the AAV captron vector preparations made without CsCl purification are likely to be 293 cell proteins that can co-purify with the AAV vector under non-ideal conditions. Regardless, the immune responses observed in the dogs that received either of these two AAV captron vector preparations were still lower than those observed in dogs that received AAV vectors made using the cap gene without CsCl purification. Therefore, those experiments with AAV captron vector preparations made without CsCl purification still revealed a reduced immunogenicity of the captron vector compared with a standard cap vector, although the magnitude of the reduction might have been larger if the two captron vector preparations did not contain the additional proteins.

All four normal dogs that received the captron vector, with or without CsCl purification, still showed some degree of immune responses as evidenced by patches of cellular infiltrates, likely due to the effects of Cap proteins present in incoming vector virions. Although further removal of empty capsid by CsCl purification or other purification techniques may minimize antigen load, immunosuppression will most likely still be required for efficient vector transduction and long-term tolerance induction. Indeed, robust transgene expression obtained in the muscles of DMD dogs in the current study suggested that a less potent immunosuppression may be sufficient to circumvent host immune responses to AAV vectors and further improve the level and duration of AAV vector-mediated transgene expression for gene therapy applications.

**MATERIALS AND METHODS**

**Animals**

Research was performed according to the principles outlined in the Guide for Laboratory Animal Facilities and Care prepared by the National Academy of Sciences, National Research Council. Dogs were housed in kennels certified by the American Association for Accreditation of Laboratory Animal Care. This study was approved by the Institutional Animal Care and Use Committee of the Fred Hutchinson Cancer Research Center, Seattle, WA, USA. All dogs were immunized for leptospirosis, distemper, hepatitis, papillomavirus and parvovirus, dewormed and observed for disease for at least 2 months before their entry into the study. Dogs weighed from 10.2 to 15.3 kg (median, 12.25 kg) and were aged between 1 and 2 years.

**AAV6 vector design and production**

For comparative studies involving normal dogs, we used an AAV vector consisting of a canine FIX cDNA driven by a CMV (cytomegalovirus) immediate early promoter and followed by a polyadenylation signal, all flanked by AAV2 terminal repeats.16–17 For studies in dystrophic dogs, we used an AAV vector consisting of a c-idades cDNA17 driven by a murine muscle creatine kinase promoter and followed by a polyadenylation signal, all flanked by AAV2 terminal repeats. For serum-neutralization studies, we used the ARAP4 AAV vector,23 which consists of a human placent al AP (alkaline phosphatase) cDNA driven by a Rous sarcoma virus promoter and followed by a polyadenylation signal, all flanked by AAV2 terminal repeats. Virus was made from the vector plasmids by four-plasmid transfection of 293 cells with the AAV vector plasmid, an AAV6 Cap expression plasmid (pcMVcap6 or pCaptron6), an AAV2 Rep expression plasmid (pTRep2) and pLadeno5 (contains adenovirus type 5 E2A, E4, and viral-associated RNA regions to provide helper functions) as outlined in Figure 1 and as previously described.18–24

**Immunosuppression, intramuscular injection and muscle biopsy**

Immunosuppression consisted of oral CSP (Teva Pharmaceuticals USA, Sellersville, PA, USA), 15 mg kg<sup>−1</sup> from day −1 to 24 weeks post injection, and subcutaneous MMF (Roche, Nutley, NJ, USA), 10 mg kg<sup>−1</sup> daily from day 0 to 24 weeks after injection. In preparation for the intramuscular injection of AAV vectors, dogs were anesthetized with isofluorane and followed by a polyadenylation signal, all flanked by AAV2 terminal repeats. Virus was made from the vector plasmids by four-plasmid transfection of 293 cells with the AAV vector plasmid, an AAV6 Cap expression plasmid (pcMVcap6 or pCaptron6), an AAV2 Rep expression plasmid (pTRep2) and pLadeno5 (contains adenovirus type 5 E2A, E4, and viral-associated RNA regions to provide helper functions) as outlined in Figure 1 and as previously described.18–24

**Histological analysis and immunofluorescence staining**

Six-μm cryostat sections were cut for analysis. For basic histological evaluation, sections were fixed in methanol, stained in hematoxylin and eosin–phloxine and mounted in VectaMount (Vector laboratories, Burlingame, CA, USA). Rabbit anti-canine FIX cDNA polyclonal antibodies (Affinity Biologicals, Ancaster, ON, Canada) and rabbit polyclonal antibodies raised against the N-terminus of mouse dystrophin (provided by Dr Jeffrey S Chamberlain, University of Washington, Seattle, WA, USA), which also detects canine dystrophin and canine μ-dystrophin, were used for immunofluorescence staining as described previously.22–27 Staining was examined and photographed using a Nikon Eclipse 800 fluorescence microscope (Nikon Instruments Inc, Melville, NY, USA).

**MRI**

Conventional <sup>1</sup>H MRI protocols were used to characterize limb skeletal muscle structure and water relaxation properties (transverse relaxation time, T2). Dogs were sedated with acepromazine (0.025 mg kg<sup>−1</sup>).
glycopyrrolate (0.011 mg kg\(^{-1}\)) and butorphanol (0.1–0.2 mg kg\(^{-1}\) IV), placed laterally on an MRI table, begun on propofol at 0.05 mg kg\(^{-1}\) min\(^{-1}\) and monitored for vital signs. Both hind limbs were taped on top of each other and inserted up to the knee with a T2 calibration standard into a two flexible element SENSE surface coil with a wider diameter of 17 cm and a less wide diameter of 13 cm (Philips Sense Flex M coil; Philips Healthcare, Best, the Netherlands) using a Philips 3 T Achieva (v 2.5 software; Philips Healthcare). The protocol consisted of T1-weighted images for fast anatomical information and a set of T2-weighted images with the recycle delay (TR) of 3000 ms and the echo time (TE) ranging from 20 to 170 ms to highlight the damaged muscle. The latter included turbo spin echo sequences to enable quantitative analysis of T2 relaxation times in the regions of injection. T2 maps were post processed by multi-echo images with 16 TE values ranging from 20 to 170 ms. The same muscle in the contralateral hind limb that was not injected served as a control. T2 values and T2-weighted images of the muscles from untreated and AAV vector-injected limbs were compared. T2 values for each inflammatory site were measured on a circle confined within a hyper-intense region in a T2 map and averaged for six consecutive slices covering the inflammatory site. Similarly, T2 values for each control site were measured either on buffer-injected sites or on the muscles with no injection. In addition, 3D T2-weighted images were acquired to cover all the injected sites using a gradient echo imaging sequence with TR of 13.8 ms, TE of 8.1 ms and flip angle of 3 degrees. Inflammatory regions were segmented from a 3D T2-weighted MR image set using a 3D Slicer (version 3.6.3 http://www.slicer.org) to delineate hyper-intense inflammatory areas. Area of each delineated inflammatory region was calculated and multiplied by its slice thickness to determine the delineated volume for the slice. Then the total volume of each inflammatory region was determined by adding all the delineated regions throughout slices corresponding to the same inflammatory site. The statistical comparisons summarized in Table 1 were made using the Prism software package version 6 (Graphpad Software, Inc., La Jolla, CA, USA).

Assay for AAV6 vector neutralization by dog serum

For the neutralization assay, 100 μl aliquots of AAV6-ARAP4 virus (10⁸ vg in Dulbecco’s modified Eagle’s medium (DMEM)) were incubated with the same volume of serial twofold diluted serum to achieve final serum dilutions of 1:2–1:5120 or were incubated with 100 μl DMEM as a negative control. The mixtures were incubated at 37 °C for 1 h, after which 80, 10, and 1 μl samples of each mixture were added to duplicate wells of HTx cells (an approximately diploid subclone of human HT-1080 fibrosarcoma cells) seeded the day before at 2 × 10⁵ cells per well in 12-well plates. The HTx cells were grown for 2–3 more days, fixed and stained for heat-stable AP expression, and AP + foci were counted to quantify the transduction rate. The neutralizing titer was defined as the reciprocal of the highest dilution of serum that inhibited AAV6 vector transduction by ≥50% compared with the untreated vector.

CONFLICT OF INTEREST

CLH and ADM are inventors on a patent application covering the captron method for AAV production. The other authors declare no conflict of interest.

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REFERENCES

1. Wang Z, Taspcott SJ, Chamberlain JS, Storb R. Immunity and AAV-mediated gene therapy for muscular dystrophies in large animal models and human trials. Front Microbiol 2011; 2: 201.
2. Rogers GL, Martinot AT, Aslani GD, Jayandharan GR, Srivastava A, Herzog RW. Innate immune responses to AAV vectors. Front Microbiol 2011; 2: 194.
3. Sabatino DE, Mingozzi F, Hui DJ, Chen H, Colosi P, Erlich HC et al. Identification of mouse AAV capsid-specific CDB + T cell epitopes. Mol Ther 2005; 12: 1033–1039.
4. Manno CS, Pierce GF, Arruda VR, Glader B, Ragni M, Rasko JL et al. Successful transduction of liver in hemophilia by AAV-factor IX and limitations imposed by the host immune response. Nat Med 2006; 12: 342–347.
5. Hauck B, Murphy SL, Smith PH, Ou G, Liu X, Zelenay O et al. Undetectable transcription of cap in a clinical AAV vector: implications for preformed capsid in immune responses. Mol Ther 2009; 17: 144–152.
6. Chadeau G, Ciron C, Moullier P, Salvetti A. Evidence for encapsidation of prokaryotic sequences during recombinant adeno-associated virus production and their in vivo persistence after vector delivery. Mol Ther 2005; 12: 744–753.
7. Halbert CL, Metzger MJ, Lam SL, Miller AD. Capsid-expressing DNA in AAV vectors and its elimination by use of an oversize capsid gene for vector production. Gene Therapy 2011; 18: 411–417.
8. Li C, Hirsch M, Asokan A, Zeithaml B, Ma H, Kafri T et al. Adeno-associated virus type 2 (AAV2) capsid-specific cytotoxic T lymphocytes eliminate only vector-transduced cells coexpressing the AAV2 capsid in vivo. J Virol 2007; 81: 7540–7547.
9. Mingozzi F, Maus MV, Hui DJ, Sabatino DE, Murphy SL, Rasko JE et al. CDB - T cell responses to adeno-associated virus capsid in humans. Nat Med 2007; 13: 419–422.
10. Wang Z, Allen JM, Riddell SR, Gregorevic P, Storb R, Taspcott SJ et al. Immunity to adeno-associated virus-mediated gene transfer in a random-bred canine model of Duchenne muscular dystrophy. Hum Gene Ther 2007; 18: 18–26.
11. Wang Z, Kuhr CS, Allen JM, Blankinship M, Gregorevic P, Chamberlain JS et al. Sustained AAV-mediated dystrophin expression in a canine model of Duchenne muscular dystrophy with a brief course of immunosuppression. Mol Ther 2007; 15: 1160–1166.
12. Wang Z, Storb R, Lee D, Kushnerick MJ, Chu B, Berger C et al. Immune responses to AAV in canine muscle monitored by cellular assays and noninvasive imaging. Mol Ther 2010; 18: 617–624.
13. Deeg HJ, Storb R, Gerhard-Miller L, Shulman HM, Weiden PL, Thomas ED. Cyclosporin A, a powerful immunosuppressant in vivo and in vitro in the dog, fails to induce tolerance. Transplantation 1980; 29: 230–235.
14. Allison AC, Eguil EM. Mycophenolate mofetil and its mechanisms of action. Immunopharmacology 2000; 47: 85–118.
15. Taylor AL, Watson CJ, Bradley JA. Immunosuppressive agents in solid organ transplantation: mechanisms of action and therapeutic efficacy. Crit Rev Oncol Hematol 2005; 56: 23–46.
16. Gregorevic P, Schultz BR, Allen JM, Haldorson JB, Blankinship MJ, Meznarich NA et al. Evaluation of vascular delivery methodologies to enhance rAAV-mediated gene transfer to canine striated muscle. Mol Ther 2009; 17: 1427–1433.
17. Wang Z, Storb R, Halbert CL, Banks GB, Butts TM, Finn EE et al. Successful regional delivery and long-term expression of a dystrophin gene in canine muscular dystrophy: a preclinical model for human therapies. Mol Ther 2012; 20: 1501–1507.
18. Wang Z, Taspcott SJ, Storb R. Local gene delivery and methods to control immune responses in muscles of normal and dystrophic dogs. Methods Mol Biol 2011; 709: 265–275.
19. Wang L, Figueredo J, Calcedo R, Lin J, Wilson JM. Cross-presentation of adeno-associated virus serotype 2 capsids activates cytotoxic T cells but does not render hepatocytes effective cytolytic targets. Hum Gene Ther 2007; 18: 185–194.
20. Van den Berghe LH, Wang L, Somananath S, Zhi Y, Figueredo J, Calcedo R et al. Heparin binding directs activation of T cells against adeno-associated virus serotype 2 capsid. Nat Med 2006; 12: 967–971.
21. Mingozzi F, Meulenberg JJ, Hui DJ, Basner-Tschakarjan E, Hasbrouck NC, Edmonson SA et al. AAV-1-mediated gene transfer to skeletal muscle in humans results in dose-dependent activation of capsid-specific T cells. Blood 2009; 114: 2077–2086.
22. Herson S, Hentati F, Rigolet A, Behin A, Romero NB, Leturcq F et al. Heparin binding directs activation of T cells against adeno-associated virus serotype 2 capsid. Nat Med 2006; 12: 967–971.