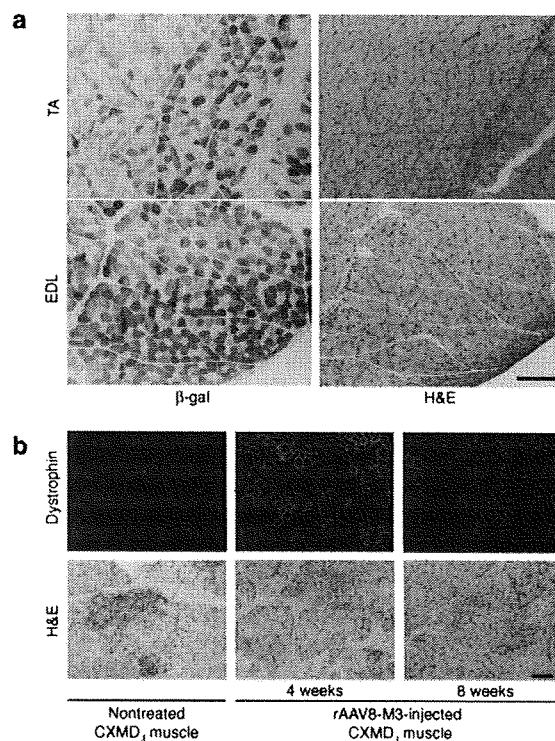


**Figure 4** Responses of dendritic cells (DCs) to rAAV in dogs. Bone marrow-derived DCs were obtained from the humerus bones of dogs and cultured in RPMI (10% FCS, p/s) for 7 days with canine GM-CSF and IL-4. **(a)** Flow cytometric analysis of cell surface molecules on day 7. The cells were stained with PE-conjugated CD11c antibody and isotype control. **(b)** DCs were stained with FITC-conjugated MHC Class II antibody and isotype control. **(c)** DCs were transduced with rAAV-*luciferase* ( $1 \times 10^6$  vg/cell) for 48 hours. To analyze luciferase expression relating to the use of rAAV2 or rAAV8, relative light unit (RLU) ratios were measured. \* $P < 0.01$ . Error bars represent s.e.m.,  $n = 8$ . **(d)** DCs were transduced with  $1 \times 10^6$  vg/cell of rAAV2 (black bars) or rAAV8-*lacZ* (gray bars) for 4 hours, and mRNA levels of MyD88, CD80, CD86, and IFN- $\beta$  were analyzed. Untransduced cells were used as a control to demonstrate the relative value of expression. The results are representative of two independent experiments. Error bars represent s.e.m.,  $n = 3$ .

seemed to be insufficient to produce the expected functional recovery (**Supplementary Figure S4c**).

For more efficient gene delivery by rAAV8, we tried a limb perfusion method in the hind limb through the lateral saphenous vein, in an attempt to prevent muscle damage due to direct injection and to bypass immune activation through DCs in the injected muscle. We had observed highly efficient  $\beta$ -gal expression in nearly all the muscles of the distal hind limb at 2 weeks after a single injection (**Table 1**, **Figure 5a**). We then injected rAAV8-*M3* into the hind limbs of CXMD<sub>1</sub> dogs, using the same method (**Table 1**). The induction of microdystrophin expression in the muscle at 4 weeks after intravascular injection was more efficient and free of noticeable immune response as compared to intramuscularly injected muscle (**Figure 5b**, **Supplementary Figure S4d**). These results suggest that the intravascular method is superior to the intramuscular method of administration. Although microdystrophin expression persisted at 8 weeks after injection of rAAV8-*M3*, the number of microdystrophin-positive cells at this time point was lower than in the muscles that were sampled at 4 weeks after injection. It is clear, therefore, that long-term microdystrophin expression can be obtained by the limb perfusion method, but that the expression does not last at the same level over a period of weeks. The same phenomenon was



**Figure 5** rAAV8-mediated muscle transduction using the limb perfusion method. **(a)** Transduction of normal dog with rAAV8-*lacZ*, using the limb perfusion method. Muscles were biopsied 2 weeks after the injection and stained with  $\beta$ -gal and H&E. TA, tibialis anterior, EDL, extensor digitorum longus. Bar = 200  $\mu$ m. **(b)** Transduction of canine X-linked muscular dystrophy in Japan (CXMD<sub>1</sub>) dog with rAAV8-*M3*. Muscles of CXMD<sub>1</sub> dogs were biopsied 4 and 8 weeks after limb perfusion with rAAV8-*M3*. Samples were immunohistochemically stained with anti-dystrophin antibody (dys2, NCL). Left: nontreated CXMD<sub>1</sub> muscle. Middle and right: muscles injected with rAAV-*M3* using limb perfusion method, examined at 4 or 8 weeks after the transduction. Bar = 100  $\mu$ m.

observed in rAAV8-*lacZ*-transduced muscles (**Supplementary Figure S5**).

## DISCUSSION

In this article, we present evidence that the transfer of rAAV8-*lacZ* to canine skeletal muscles produces higher transgene expression with less lymphocyte proliferation than rAAV2-*lacZ* does, at 2 weeks after injection. Given the advantages of rAAV8, the administration of rAAV8-*M3* by limb perfusion produced extensive transgene expression in the distal limb muscles of CXMD<sub>1</sub> dogs without obvious immune responses for as long as 8 weeks after injection. However, transgene expression in the rAAV8-transduced muscles attenuated in the absence of an immunosuppressive regimen over the course of observation. In addition, humoral immune responses were elicited by both rAAV2 and rAAV8. mRNA levels of MyD88 and costimulating factors such as CD80, CD86, and type I interferon (interferon- $\beta$ ) were elevated in both rAAV2- and rAAV8-transduced DCs *in vitro*.

In our previous study, we had demonstrated extensive lymphocyte-mediated immune responses to rAAV2-*lacZ* after direct intramuscular injection into dogs, in contrast to the reported successful delivery of the same viral construct into mouse skeletal

muscle.<sup>8</sup> The fact that the promoter-deleted rAAV2 caused fewer cytotoxic cellular responses suggested that the massive destruction of transduced muscle cells might be the result of cellular immunity against the transgene product. In this study, there was extensive expression of  $\beta$ -gal in rAAV8-*lacZ*-injected canine muscles even in the absence of any immunosuppressive treatments (Figure 1), while the rAAV2-*lacZ*-injected muscles showed minimal  $\beta$ -gal expression with considerable inflammatory infiltration. If the transgene product were the main inducer of immune responses, lymphocyte activation would be correlated with transduction efficiency; however, this is not the case based on our results relating to the vector genome, mRNA expression level, and protein delivered through either rAAV2 or rAAV8 (Figure 2). These data suggested that the rAAV particle is associated with potent immunogenicity. Besides,  $\beta$ -gal expression disappeared 4 weeks after injection in the rAAV8-injected muscle as in the rAAV2-transduced muscles (Supplementary Figure S2). To investigate whether AAV itself has immunogenicity properties, we further characterized the immune responses caused by rAAV2 or rAAV8.

Immunohistochemical analysis revealed that the rAAV2-injected muscles showed higher rates of infiltration of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes in the endomysium than rAAV8-injected muscles did (Figure 3a). Considering the stringent immunogenicity of *lacZ* gene expression, we normalized the activity of TGF- $\beta$ 1 and IL-6 by *lacZ* expression to exclude the effect of transgene products (Supplementary Figure S3a). The total activity of TGF- $\beta$ 1 and IL-6 in the rAAV8-injected muscles was higher than that in rAAV2-injected muscles (Supplementary Figure S3b). As a result, rAAV2 induced a stronger cellular immune response than rAAV8 did. To investigate the humoral immune response, we quantitated neutralizing antibodies against rAAV particles in the sera of rAAV-injected dogs (Figure 3b). Antibodies against AAV2 and AAV8 capsids were below the detectable level before the injection and were elevated with time after the injection. Because the dogs were bred in a specific pathogen-free facility and not vaccinated, we assume that the elevation of antibody levels was not caused by anamnestic reaction.

Recently, Li *et al.*<sup>10</sup> reported that the AAV2 capsid can induce a cellular immune response through MHC class I antigen presentation with a cross-presentation pathway, and the effects of rAAV2 on human DCs have been described.<sup>10,13</sup> In contrast, other serotypes such as rAAV8 induced less T-cell activation.<sup>11,14</sup> Plasmacytoid DCs are critically important in innate immunity because of their unsurpassed ability to present adenoviral antigens to T-cells for the generation of primary cellular and humoral immune responses.<sup>15-17</sup> The response of DCs against rAAV in dogs was yet to be elucidated. We prepared bone marrow-derived DCs to investigate rAAV-mediated transduction of DCs. The difference between rAAV2 and rAAV8 in respect of the transduction rate of DCs *in vitro* was no greater than the difference in distinct  $\beta$ -gal expressions *in vivo* (Figure 2,4c). Quantitative analysis of mRNA of the transduced DCs by RT-PCR revealed that both rAAV2 and rAAV8 upregulated the expression of costimulating factors, with no significant difference between mRNA levels in rAAV2- and rAAV8-transduced cells. Therefore, both rAAV2 and rAAV8 may activate innate immunity in the context of extensive muscle transduction. Whereas AAV capsids cause immune

response, transgene products may play adjuvant roles in the immunity to the AAV capsids.<sup>18</sup>

rAAV8 encoding the human *microdystrophin* gene was also intramuscularly injected into the skeletal muscles of CXMD<sub>1</sub> dogs. rAAV8-mediated gene expression without any immunosuppression was confirmed over a period of 8 weeks after the injection, whereas there was much less transduction with the use of rAAV2 (data not shown). rAAV8-mediated transduction was also expected to provide effective intravenous delivery.<sup>12</sup> In this context, the venous system is an attractive route for limb perfusion administration because it is a direct channel to multiple muscles of the limb. Moreover, veins are easier to access through the skin and there is less potential for muscle damage during injection. By using the limb perfusion method, we could reach nearly all the muscles of the lower limb, held transiently isolated by a tourniquet around the thigh. Limb perfusion administration could possibly have the potential to bypass the DC recognition caused by intramuscular injection. We intravenously injected rAAV8-*lacZ* into the hind limbs of normal dogs and rAAV8-*M3* into the hind limbs of CXMD<sub>1</sub> dogs, and obtained more extensive expression of  $\beta$ -gal or *microdystrophin* than by intramuscular injection. Interestingly, the inflammatory response was not significant in the intravenously injected muscles, although no immune suppression was attempted. We think that one reason rAAV8-*M3* resulted in better expression than rAAV8-*lacZ* is that the immunogenicity of *M3* is lower than that of *lacZ*. Although *microdystrophin* expression was lower at 8 weeks after the transduction with the limb perfusion, cellular infiltration was not significant.

In the future, systemic delivery of rAAV8-*microdystrophin* could ameliorate the symptoms of DMD patients. Even though portal vein injection of rAAV2-*FIX* into hemophilia B dogs produced long-term expression, a clinical study failed to demonstrate long-term expression in humans.<sup>19,20</sup> In advance of future clinical trials, several studies are required to confirm safety. Sequential peripheral blood monitoring showed no severe adverse events, including liver dysfunction, during 8 weeks (data not shown). We are now developing a systemic delivery strategy with a muscle-specific promoter. It is also necessary to improve vector constructs or regulate immune reaction against transgene products. Recently, Wang *et al.* reported sustained AAV6-mediated human *microdystrophin* expression in dystrophic dogs for 30 weeks, using combined immunosuppressive therapy of Cyclosporin, Mycophenolate Mofetil, and anti-thymocyte globulin.<sup>9</sup> In this study with rAAV8-*M3*, we confirmed effective transduction into dog skeletal muscle for 4 weeks without immunosuppressive therapy. However, considering the fact that not only rAAV2 but also rAAV8 induced activation of DCs *in vitro*, immunological modulation would be required for sufficient long-term expression. A novel protocol with systemic or localized immunosuppression using immunosuppressive drugs or local immunosuppression with an IFN- $\alpha$  or - $\beta$  blockade could help avoid host immune reaction.

In summary, we achieved successful rAAV8-mediated muscle transduction in wild-type dogs as well as in dystrophic dogs by using the limb perfusion method of administration. Also, by manipulating bone marrow-derived DCs, we observed the probable contribution of antigen-presenting cells to the immune response against rAAV8-mediated gene therapy. Although the

cellular responses against rAAV8 were not significant *in vivo*, this DC activation may possibly be involved in limiting long-term transduction when the limb perfusion method is used. The limb perfusion transduction protocol with improved AAV constructs or immune modulation would further enhance rAAV8-mediated transduction strategy and lead to therapeutic benefits.

## MATERIALS AND METHODS

**Animals.** Five- to ten-week-old male and female wild-type dogs obtained from the Beagle-based CXMD<sub>1</sub> breeding colony at the National Center of Neurology and Psychiatry (Tokyo, Japan) were used for the *lacZ* gene transduction.<sup>3</sup> Six- to eight-week-old CXMD<sub>1</sub> dogs were used for *microdystrophin* gene transduction. All the animals were cared for and treated in accordance with the guidelines approved by the Ethics Committee for Treatment of Laboratory Animals at National Center of Neurology and Psychiatry, where the three fundamental principles of replacement, reduction, and refinement are also considered. Dogs were not vaccinated to avoid the immune responses to vaccination.

**Construction of proviral plasmid and recombinant AAV vector production.** The AAV2 vector proviral plasmids harboring the *lacZ* or *luciferase* gene with a CMV promoter and SV40 late-gene polyadenylation sequence were propagated.<sup>8</sup> As a therapeutic gene for DMD, the human *microdystrophin* gene, *M3*, was used under the control of the CMV promoter and a bovine growth hormone polyadenylation sequence.<sup>21</sup> The vector genome was packaged into the AAV2 capsid or pseudotyped AAV8 capsid in HEK293 cells. A large-scale cell culture method with an active gassing system was used for transfection.<sup>22</sup> The vector production process involved triple transfection of a proviral plasmid, an AAV helper plasmid pAAV-RC (Stratagene, La Jolla, CA) or p5E18-VD2/8, and an adenovirus helper plasmid pHelper (Stratagene).<sup>21</sup> All the viral particles were purified by CsCl gradient centrifugation. The viral titers were determined by quantitative PCR using SYBR-green detection of PCR products in real time with the MyiQ single-color detection system (Bio-Rad, Hercules, CA) and the following primer sets: for AAV-*lacZ*, *lacZ*-Q60: forward primer 5'-TTATCAGCCGGAAAACCTACCG-3', and reverse primer 5'-AGCCAGTTTACCCGCTCTGCTA-3'; for AAV-*microdystrophin*: forward primer 5'-CCAAAAGAAAAGGATCCACAA-3', and reverse primer 5'-TTCCAAATCAAACCAAGAGTCA-3'; and for AAV-*luciferase*: forward primer 5'-GATACGCTGCTTAAATGCCTTT-3', and reverse primer 5'-GTTGCGTCAGCAAACACAGT-3'.

**Direct administration of rAAVs into normal and dystrophic skeletal muscle.** Experimental dogs ( $n = 16$ ) were sedated with isoflurane by mask inhalation and intubated. Anesthesia was maintained with 2–4% isoflurane. Two milliliters of rAAV2-*lacZ* or rAAV8-*lacZ* ( $1 \times 10^{11}$ – $10^{13}$  vg/ml) were injected intramuscularly into the tibialis anterior muscles and 1 ml into the extensor carpi radialis muscles of the normal dogs under ultrasonographic guidance. rAAV8-*M3* ( $1 \times 10^{12}$  vg/ml) was intramuscularly injected at a volume of 2 ml into the tibialis anterior muscles and 1 ml into the extensor carpi radialis muscles of a CXMD<sub>1</sub> dog.

**Intravenous delivery of rAAVs into the limb veins of dogs.** Intravenous injection was administered as described elsewhere.<sup>12</sup> Briefly, a blood pressure cuff was applied just above the knee of an anesthetized normal dog. A 24-gauge intravenous catheter was inserted into the lateral saphenous vein, connected to a three-way stopcock, and flushed with saline. With the blood pressure cuff inflated to over 300 mm Hg, saline (2.6 ml/kg) containing papaverine (0.44 mg/kg, Sigma-Aldrich, St Louis, MO) and heparin (16 U/kg) was injected by hand over 10 seconds. The three-way stopcock was connected to a syringe containing rAAV8-*lacZ* ( $1 \times 10^{14}$  vg/kg, 3.8 ml/kg). The syringe was placed in a PHD 2000 syringe pump (Harvard Apparatus, Edenbridge, UK). Five minutes after the

papaverine/heparin injection, the rAAV8-*lacZ* was injected at a rate of 0.6 ml/second. Two minutes after the rAAV injection, the blood pressure cuff was released and the catheter was removed. The CXMD<sub>1</sub> dogs were injected with rAAV8-*M3* using the same method.

**Sampling of transduced muscles.** Either the muscles of the transduced dogs were biopsied or the animals were killed at 2, 4, and 8 weeks after the injection. We sampled tibialis anterior and extensor carpi radialis muscles on both sides in the intramuscularly transduced dog. In the case of the limb perfusion study, tibialis anterior or extensor digitorum longus muscle of the injected side of the leg was sampled. For biopsy and necropsy, the individual muscle was cropped tendon-to-tendon, divided into several pieces, and immediately frozen in liquid nitrogen-cooled isopentane. Two to eight blocks were sampled from the transduced muscle. We analyzed at least 30 sections from the blocks to observe the general representation.

**Histological analysis.** Transverse cryosections (10  $\mu$ m) from the rAAV-*lacZ*-injected muscles were stained with hematoxylin and eosin or 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside.<sup>23</sup> Eight-micrometer-thick cryosections from the rAAV-*M3*-injected muscles were immunohistochemically stained as described.<sup>24</sup> Briefly, the cryosections were fixed by immersion in cold acetone at  $-20^{\circ}\text{C}$ . Fixed frozen sections were blocked in 5% goat serum in phosphate-buffered saline at room temperature and incubated with mouse monoclonal anti-dystrophin C-terminal antibody (NCL-dys2, Novocastra, Newcastle upon Tyne, UK). The signal was visualized with an Alexa 568-conjugated anti-mouse IgG. Fluorescent signals were observed using a confocal laser scanning microscope (Leica TCS SP, Leica, Heidelberg, Germany). Immunohistochemical analyses were performed with mouse monoclonal antibodies against canine CD4 (CA13.1E4, Serotec, Oxford, UK), canine CD8a (CA9.1D3, Serotec), and double-stained with rabbit polyclonal antibody against  $\alpha$ -sarcoglycan.<sup>25</sup> The signal was visualized with an Alexa 568-conjugated anti-mouse IgG, and 488-conjugated anti-rabbit IgG.

**Detection of AAV genomes.** Total DNA was extracted from muscle cryosections. Cryosections were homogenized using a Multi-beads shocker (Yasui Kikai, Osaka, Japan), and extracted using a Wizard SV Genomic DNA purification system (Promega, Madison, WI). The rAAV genome was detected by relative quantitative PCR using SYBR-green detection of PCR products in real time with a primer set of *lacZ*-Q60. For an internal control, forward primer, 5'-GAACACGCGTTAATAAGGCAATCA-3', and reverse primer, 5'-CTGACATTCATCGCATCTTTGACA-3', directed to an ultra-conserved region, were used.<sup>26</sup>

**Real-time RT-PCR.** Total RNA was isolated from cryosections using a Multi-beads shocker (Yasui Kikai), and RNeasy Fibrous Tissue Mini kit (Qiagen, Hilden, Germany), and first-strand cDNA was synthesized using a QuantiTect Reverse Transcription kit (Qiagen). mRNA was detected using primer sets of *lacZ*-Q60, forward primer 5'-TGATGGCTA CTGCTTCCCTAC-3' and reverse primer 5'-GAGATTTGCCGA GGATGTACT-3' for IL-6, and forward primer 5'-CAAGGATCTGGGC TGGAAAGTGGA-3' and reverse primer, 5'-CCAGGACCTTGCTGTA CTGCGGT-3' for TGF- $\beta$ 1. For an internal control, a primer set of 18S rRNA (Ambion, Foster City, CA) was used.

**Western blot analysis.** Muscle cryosections were homogenized with four volumes of sample buffer (10% SDS, 70 mmol/l Tris-HCl, 10 mmol/l EDTA, and 5%  $\beta$ -mercaptoethanol). The samples were boiled for 5 minutes and centrifuged at 14,500 rpm for 15 minutes. Protein samples (30  $\mu$ g per lane) were electrophoresed on a 7.5% polyacrylamide gel (Bio-Rad). The membranes were incubated with a 1:1,000 dilution of the primary antibody for detecting 120 kDa *lacZ* protein (rabbit anti- $\beta$ -galactosidase IgG fraction, Molecular Probes, Eugene, OR) or 42 kDa  $\alpha$ -actin (mouse anti- $\alpha$ -sarcomeric actin IgM, Sigma-Aldrich). Anti-rabbit IgG peroxidase F(ab')

(GE Healthcare, Buckinghamshire, UK), or peroxidase-conjugated donkey anti-mouse IgM (Jackson ImmunoResearch Laboratories, West Grove, PA) was used for ECL immunodetection (GE Healthcare). Quantification of LacZ protein was performed using a specialized software (ImageJ, US National Institutes of Health, Bethesda, MD).

**ELISA for anti-canine AAV IgG.** A microtiter plate (MS-8596F, Sumitomo Bakelite, Tokyo, Japan) was precoated with promoter-deleted rAAV2 or rAAV8 ( $2 \times 10^9$  genomes/well) and blocked with a blocking buffer (Block Ace, DS Pharma Biomedical, Osaka, Japan). The plate was incubated for 2 hours at room temperature with the sera from rAAV-transduced dogs, followed by a 1:5,000 dilution of peroxidase-conjugated rabbit anti-dog IgG (Sigma-Aldrich) for 1 hour. Color was visualized using a peroxidase substrate system (TMBZ, ML-1120T, Sumitomo Bakelite). Reactivity was detected at a wave-length of 450 nm with a reference at 570 nm, using an APPLISKAN Multimode Reader (Thermo Fisher Scientific, East Greenbush, NY).

**Bone marrow aspiration and preparation of DCs.** After the dogs were anesthetized with thiopental and isoflurane, ~0.5 ml of bone marrow was obtained from each humerus by aspiration with a syringe containing 2 ml of 16 mmol/l EDTA-2Na PBS. Bone marrow-derived DCs were generated as described.<sup>15</sup> Mononuclear cells were isolated by density centrifugation using Histopaque-1077 (Sigma-Aldrich). Cells were suspended in RPMI-1640 culture medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (MP Biomedicals, Aurora, OH) and 1% penicillin-streptomycin (Sigma-Aldrich), and cultured at 37°C in a humidified 5% CO<sub>2</sub>-containing atmosphere. Recombinant canine GM-CSF (25 ng/ml, R&D Systems, Minneapolis, MN) and canine IL-4 (12.5 ng/ml, R&D Systems) were added to the culture medium. On days 3 and 5 of the culture, 60% of the medium volume was changed. On day 7 of the culture, loosely adherent cells were collected and used for fluorescence-activated cell analysis. A FACS Vantage system (Becton Dickinson, Franklin Lakes, NJ) was used for flow cytometry event collection. For the purpose of examining the infectious rate of rAAV, cells were cultured for 48 hours with rAAV2- or 8-*luciferase*. The luciferase activity of rAAV2- or rAAV8-*luciferase* co-cultured cells was estimated using an APPLISKAN Multimode Reader (Thermo Fisher Scientific). Total RNA was isolated using an RNeasy Fibrous Tissue Mini kit (Qiagen), and QuantiTect Reverse Transcription kit (Qiagen). mRNA of cytokines were analyzed using the primer set, forward primer 5'-GAGGAGATGGGCTTCGAGTA-3' and reverse primer 5'-GTTCCACCAACAGCTCGTC-3' for MyD88; forward primer 5'-GCATCATCCAGGTGAACAAG-3' and reverse primer 5'-AAGTCAGCAAAGGTGCGATT-3' for CD80; forward primer 5'-AGGTTACCCAGAACCAAGG-3' and reverse primer, 5'-TTGCAGGACACAGAAGATGC-3' for CD86; and forward primer 5'-ATTGCCTCAAGGACAGGATAAAA-3' and reverse primer 5'-TTGACGTCCTCCAGGATTATCT-3' for IFN- $\beta$ . mRNA levels of MyD88, CD80, CD86, and IFN- $\beta$  in DCs were normalized with a house keeping gene, 18s rRNA. The mRNA levels in the transduced cells were presented as ratios relative to the sample obtained from the untransduced DCs.

**Statistical analysis.** Statistical significance was determined on the basis of an unpaired, two-tailed Student's *t*-test using specialized software (Statview; SAS Institute, Cary, NC). A *P* value of <0.05 was considered significant.

#### SUPPLEMENTARY MATERIAL

**Figure S1.** Histological findings with incisional and nonincisional injection under ultrasonographic guidance.

**Figure S2.**  $\beta$ -gal expression 4 weeks after injection.

**Figure S3.** Levels of mRNA were investigated using rAAV-injected muscles.

**Figure S4.** Intramuscular injection of rAAV8-M3 into CXMD<sub>1</sub>

**Figure S5.** Long-term  $\beta$ -gal expression using limb perfusion injection.

**Table S1.** Protein expression analyzed with ImageJ.

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#### REFERENCES

- Valentine, BA, Cooper, BJ, de Lahunta, A, O'Quinn, R and Blue, JT (1988). Canine X-linked muscular dystrophy. An animal model of Duchenne muscular dystrophy: clinical studies. *J Neurol Sci* **88**: 69–81.
- Sharp, NJ, Kornegay, JN, Van Camp, SD, Herbstreith, MH, Secore, SL, Kettle, S et al. (1992). An error in dystrophin mRNA processing in golden retriever muscular dystrophy, an animal homologue of Duchenne muscular dystrophy. *Genomics* **13**: 115–121.
- Shimatsu, Y, Yoshimura, M, Yuasa, K, Urasawa, N, Tomohiro, M, Nakura, M et al. (2005). Major clinical and histopathological characteristics of canine X-linked muscular dystrophy in Japan, CXMD<sub>1</sub>. *Acta Myol* **24**: 145–154.
- Nakai, H, Fuess, S, Storm, TA, Muramatsu, S, Nara, Y and Kay, MA (2005). Unrestricted hepatocyte transduction with adeno-associated virus serotype 8 vectors in mice. *J Virol* **79**: 214–224.
- Wang, Z, Zhu, T, Qiao, C, Zhou, L, Wang, B, Zhang, J et al. (2005). Adeno-associated virus serotype 8 efficiently delivers genes to muscle and heart. *Nat Biotechnol* **23**: 321–328.
- Yoshimura, M, Sakamoto, M, Ikemoto, M, Mochizuki, Y, Yuasa, K, Miyagoe-Suzuki, Y et al. (2004). AAV vector-mediated microdystrophin expression in a relatively small percentage of mdx myofibers improved the mdx phenotype. *Mol Ther* **10**: 821–828.
- Gregorevic, P, Allen, JM, Minami, E, Blankinship, MJ, Haraguchi, M, Meuse, L et al. (2006). rAAV6-microdystrophin preserves muscle function and extends lifespan in severely dystrophic mice. *Nat Med* **12**: 787–789.
- Yuasa, K, Yoshimura, M, Urasawa, N, Ohshima, S, Howell, JM, Nakamura, A et al. (2007). Injection of a recombinant AAV serotype 2 into canine skeletal muscles evokes strong immune responses against transgene products. *Gene Ther* **14**: 1249–1260.
- Wang, Z, Kuhr, CS, Allen, JM, Blankinship, M, Gregorevic, P, Chamberlain, JS et al. (2007). Sustained AAV-mediated dystrophin expression in a canine model of Duchenne muscular dystrophy with a brief course of immunosuppression. *Mol Ther* **15**: 1160–1166.
- Li, C, Hirsch, M, Asokan, A, Zeithaml, B, Ma, H, Kafri, T et al. (2007). Adeno-associated virus type 2 (AAV2) capsid-specific cytotoxic T lymphocytes eliminate only vector-transduced cells coexpressing the AAV2 capsid *in vivo*. *J Virol* **81**: 7540–7547.
- Vandenbergh, LH, Wang, L, Somanathan, S, Zhi, Y, Figueredo, J, Calcedo, R et al. (2006). Heparin binding directs activation of T cells against adeno-associated virus serotype 2 capsid. *Nat Med* **12**: 967–971.
- Hagstrom, JE, Hegge, J, Zhang, G, Noble, M, Budker, V, Lewis, DL et al. (2004). A facile nonviral method for delivering genes and siRNAs to skeletal muscle of mammalian limbs. *Mol Ther* **10**: 386–398.
- Zhang, Y, Chirmule, N, Gao, G and Wilson, J (2000). CD40 ligand-dependent activation of cytotoxic T lymphocytes by adeno-associated virus vectors *in vivo*: role of immature dendritic cells. *J Virol* **74**: 8003–8010.
- Lin, SW, Hensley, SE, Tatsis, N, Lasaro, MO and Ertl, HC (2007). Recombinant adeno-associated virus vectors induce functionally impaired transgene product-specific CD8 T cells in mice. *J Clin Invest* **117**: 3958–3970.
- Isotani, M, Katsuma, K, Tamura, K, Yamada, M, Yagihara, H, Azakami, D et al. (2006). Efficient generation of canine bone marrow-derived dendritic cells. *J Vet Med Sci* **68**: 809–814.
- Zhu, J, Huang, X and Yang, Y (2007). Innate immune response to adenoviral vectors is mediated by both Toll-like receptor-dependent and -independent pathways. *J Virol* **81**: 3170–3180.
- Zhang, Z and Wang, FS (2005). Plasmacytoid dendritic cells act as the most competent cell type in linking antiviral innate and adaptive immune responses. *Cell Mol Immunol* **2**: 411–417.
- Mahadevan, M, Liu, Y, You, C, Luo, R, You, H, Mehta, JL et al. (2007). Generation of robust cytotoxic T lymphocytes against prostate specific antigen by transduction of dendritic cells using protein and recombinant adeno-associated virus. *Cancer Immunol Immunother* **56**: 1615–1624.
- Mount, JD, Herzog, RW, Tillson, DM, Goodman, SA, Robinson, N, McClelland, ML et al. (2002). Sustained phenotypic correction of hemophilia B dogs with a factor IX null mutation by liver-directed gene therapy. *Blood* **99**: 2670–2676.

20. Manno, CS, Pierce, GF, Arruda, VR, Glader, B, Ragni, M, Rasko, JJ *et al.* (2006). Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat Med* **12**: 342–347.
21. Sakamoto, M, Yuasa, K, Yoshimura, M, Yokota, T, Ikemoto, T, Suzuki, M *et al.* (2002). Micro-dystrophin cDNA ameliorates dystrophic phenotypes when introduced into mdx mice as a transgene. *Biochem Biophys Res Commun* **293**: 1265–1272.
22. Okada, T, Nomoto, T, Yoshioka, T, Nonaka–Sarukawa, M, Ito, T, Ogura, T *et al.* (2005). Large-scale production of recombinant viruses by use of a large culture vessel with active gassing. *Hum Gene Ther* **16**: 1212–1218.
23. Ishii, A, Hagiwara, Y, Saito, Y, Yamamoto, K, Yuasa, K, Sato, Y *et al.* (1999). Effective adenovirus-mediated gene expression in adult murine skeletal muscle. *Muscle Nerve* **22**: 592–599.
24. Yuasa, K, Sakamoto, M, Miyagoe-Suzuki, Y, Tanouchi, A, Yamamoto, H, Li, J *et al.* (2002). Adeno-associated virus vector-mediated gene transfer into dystrophin-deficient skeletal muscles evokes enhanced immune response against the transgene product. *Gene Ther* **9**: 1576–1588.
25. Araishi, K, Sasaoka, T, Imamura, M, Noguchi, S, Hama, H, Wakabayashi, E *et al.* (1999). Loss of the sarcoglycan complex and sarcospan leads to muscular dystrophy in beta-sarcoglycan-deficient mice. *Hum Mol Genet* **8**: 1589–1598.
26. Sandelin, A, Bailey, P, Bruce, S, Engstrom, PG, Klos, JM, Wasserman, WW *et al.* (2004). Arrays of ultraconserved non-coding regions span the loci of key developmental genes in vertebrate genomes. *BMC Genomics* **5**: 99.



