

Transient Immunomodulation Allows Repeated Injections of AAV1 and Correction of Muscular Dystrophy in Multiple Muscles

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Exon-skipping AAV1-U7-associated therapy is a promising treatment for Duchenne muscular dystrophy (DMD). We have shown earlier that the newly rescued dystrophin protein is stably expressed for months in mice and dogs, and does not induce immune rejection of transduced fibers. In this study, we used the dystrophic *mdx* mouse as a preclinical model to characterize the immune response to the adeno-associated virus 1 (AAV1) vector, and tested the feasibility of administering multiple AAV1 injections to extend the treatment to several muscles. We found that re-injections of AAV1 vector are compromised as early as 3 days after the first injection, coincident with a rapid increase in AAV1-specific immunoglobulin M (IgM) and IgG in the serum. Adoptive transfer of immune sera confirmed the rapid appearance of an AAV1 neutralization activity, and experiments with immunoglobulin-deficient (μ KO) mice proved that antibodies (Abs) are the only effectors responsible for AAV1-U7 elimination. It is important to note, however, that the AAV2 vector still generated an adverse immune response in μ KO mice. By blocking the T-B crosstalk with anti-CD40 Abs and CTLA4/Fc fusion protein, we found that a mere 5 days of immunomodulation treatment was sufficient to totally abrogate the formation of anti-AAV1 Abs and to allow for the correction of muscular dystrophy in multiple muscles, provided the treatment was administered during each challenge.

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INTRODUCTION

Mutations in the dystrophin gene cause Duchenne muscular dystrophy (DMD), the most common severe childhood muscular pathology. There is currently no effective treatment for the condition. With antisense sequences linked to a modified U7 small

nuclear RNA, we achieved persistent exon skipping that removes the mutated exon and re-establishes the open reading frame on the dystrophin messenger RNA of the animal models for DMD, the *mdx* mouse, and golden retriever muscular dystrophy dog.^{1,2} In the treated muscles, dystrophin expression, muscle morphology amelioration, and significant force recovery were obtained. This successful dystrophin rescue by AAV1-U7 therapy is partly explained by the high tropism of AAV1 (adeno-associated virus 1) for muscle.³ Yet most clinical trials so far have been performed using AAV2-derived vectors (for review see ref. 4).

AAV1-U7 treatment aimed at restoring muscle function body-wide will likely require multiple re-injections. There are at least two reasons for this. First, although AAV vector genomes clearly persist in muscles for several years,⁵ the beneficial expression of the antisense may disappear with time, given that there is no evidence of AAV vector genome integration into host chromosomes in either fibers or myogenic stem cells.⁶ Second, despite the significant progress in recent times in body-wide vector delivery to striated muscles and hearts of mice,^{7–9} the translation of this approach to large animals and humans is challenging because of the high doses of vector required.

When the commonly used AAV2 vector was employed in preclinical models, little or no transduction was detected after the second injection. The presence of neutralizing antibodies (Abs) to AAV2 capsid proteins resulting from the first vector injection (as detected in *in vitro* studies) appears to be the cause of the poor transduction after the second injection.^{10–12} Furthermore, the results of a completed hemophilia B clinical trial have revealed that specific cytotoxic T-lymphocyte (CTL) responses targeting epitopes of the AAV2 capsid protein can limit the duration of therapeutically relevant transgene expression.¹³ These CTL responses to AAV2 capsids were further analyzed in healthy donors, and can be attributed to the reactivation of memory T cells.¹⁴ Similar observations in mice have been reported,^{15,16} and it was shown, in particular, that the AAV2 VP3 protein contains a heparan sulfate proteoglycan RXXR-binding motif which directs

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T-cell activation.¹⁷ This VP3 heparan sulfate proteoglycan-binding motif is not present in other AAV serotypes such as AAV8 (ref. 17) and AAV1 (ref. 18).

Because of its ribonucleotidic nature U7 small nuclear RNA is not immunogenic *per se*, and its expression induces long-term expression of a truncated but functional dystrophin in mice and dogs.¹² This shortened dystrophin is similar to the one observed in revertant dystrophin-positive fibers in the muscles of human DMD patients and *mdx* mice in an otherwise dystrophin-negative background.^{19,20} This would explain the lack of immune response against the rescued dystrophin, in contrast to restoration of full-length dystrophin.²¹ Moreover, although the nature of immune response to AAV1 vectors has not been investigated, it has been shown in mice that, when re-injected into muscle, AAV1 vectors encounter an adaptative immune response that blocks subsequent gene delivery with the same brand of vector.^{22,23}

A thorough understanding of this adaptive response following the use of AAV1-U7-based treatment in diseased muscles of dystrophic mice is crucial for the design of future gene therapy clinical trials, given that the first exposure to the vector could preclude further treatment with the same vector, particularly in the microenvironment of dystrophic muscle which undergoes constant regeneration of fragile fibers.

Although we know that targeting of the whole skeletal musculature would require systemic delivery of AAV vectors, early clinical trials most often use the intramuscular route. This is unlikely to be broadly and repetitively applicable for any wide-spread benefit in patients. In this study, using dystrophic mice, we evaluated the immunological consequences of intramuscular injections of AAV1 vectors administered for the purpose of rescuing a tolerated quasi-dystrophin by exon skipping. We found that neutralizing Abs against the vector itself were the only effectors responsible for the failure of efficacy of AAV1 re-injection in mice. We showed that transient inhibition of co-stimulatory molecules during the period of AAV1 delivery, by using anti-CD40 Abs and CTLA4/Fc fusion protein, overcame this immune response and allowed sequential treatments and the consequent rescue of dystrophin in multiple muscles.

RESULTS

Impairment of dystrophin rescue after secondary AAV1-U7 challenge

AAV1-U7 treatment requires several injections in different sites to rescue the maximum possible number of muscles. We initially evaluated the effect of sequential challenges in *mdx* mice by injecting AAV1-U7 first in the *tibialis anterior* (TA) muscle and 15 days later in the contralateral TA muscle. Whereas the first injected TA exhibited a strong and sustained dystrophin expression, underlining the lack of immune response against the rescued dystrophin, the contralateral injected muscle exhibited the characteristics of an untreated muscle (Figure 1a and b), thereby demonstrating that the second AAV1-U7 injection was ineffective. In view of the finding that the first injected muscle exhibited a territory of dystrophin expression as extensive as would be expected after a single AAV1-U7 injection, we hypothesized that the ineffectiveness of subsequent AAV1-U7 injections resulted from an anti-AAV1 specific immune response.

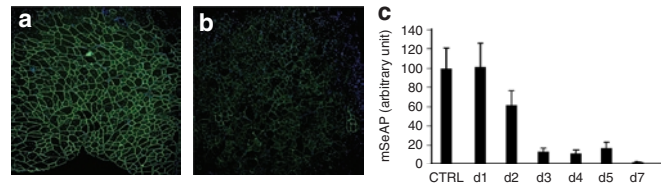


Figure 1 Second injections of adeno-associated virus 1 (AAV1) particles in *tibialis anterior* (TA) are ineffective after 3 days. *mdx* mice were injected in the TA with 10^{11} AAV1-U7 viral genomes (vg) at (a) day 0, and (b) 15 days later in the contralateral TA. Thirty days after the latest injection, dystrophin rescue was monitored by NCL-DYS2 immunostaining on whole transverse sections of TA muscles. One representative immunostained section of five different muscles is shown for each condition. (c) B6 mice were injected in the TA muscle with 10^{11} AAV1-U7 vg at day 0 and in the contralateral TA muscle with AAV1-mSeAP (10^{10} vg) at days 1–7 (d1–d7). Serum levels of murine secreted alkaline phosphatase (mSeAP) were measured 15 days after AAV1-mSeAP injection. The control (CTRL) corresponds to AAV1-mSeAP expression level resulting from a single injection of AAV1-mSeAP (10^{10} vg) used as normalization for 100% of the transduction efficiency. The data represent the mean values of three mice per group \pm SEM. One of two representative experiments is shown.

In order to test the immunogenicity of the AAV1 capsid itself, we set up an experimental system in which only the AAV1 capsid was shared between two successive injections, using the murine secreted alkaline phosphatase (mSeAP) (a non-immunogenic self-protein that can be detected in serum) as a reporter transgene for the second challenge. Given that spontaneous self renewal of dystrophic muscle drastically reduces mSeAP activity until it almost completely disappears,²⁴ we carried out these experiments in normal C57BL/6 mice (B6). This allowed for an appraisal of the kinetics of rejection of the second challenge independent of the expected loss of mSeAP activity connected with the ongoing dystrophic process. B6 mice were intramuscularly injected at day 0 with 10^{11} AAV1-U7 vg as previously described, and subsequently received 10^{10} AAV1-mSeAP vg in contralateral muscles alternately from day 1 to day 7. This dosage of AAV1-mSeAP allowed robust and reproducible levels of alkaline phosphatase activity in sera and/or tissue sections. In groups of mice re-injected on day 3, the mSeAP serum level dipped to 10% of the AAV1-mSeAP expression after a single injection, and was undetectable after a week (Figure 1c). These experiments suggest that mice challenged with AAV1 in TA raise an immune response directed against the AAV1 capsid within 1 week, preventing successful AAV1 re-injection. The transduction efficiency of the second challenge using AAV1-mSeAP seemed, therefore, to be limited by the magnitude of the anti-AAV1 immune response. Remarkably, no signs of cell-death or muscle-tissue renewal were detected at the secondary sites, thereby suggesting the absence of muscle-fiber destruction by either complement or cytotoxic activity.

Humoral response against AAV1 capsid

It is well known that the lack of dystrophin triggers muscle-fiber micro-damage spread over the whole skeletal musculature. Scavenger cells potentially involved in the immune system, such as macrophages, home in toward these sites of necrosis, provoking the inflammation-like reactions that are essential for local muscle repair. Yet, there is no evidence that these circumstances lead to immunological problems or hypersensitivity in dystrophic individuals (*mdx*

mice, golden retriever muscular dystrophy dogs, and human DMD patients). In order to confirm this, we first compared the humoral responses against AAV1 in *mdx* and normal B6 mice after intramuscular injection of 10^{11} AAV1-U7 vg (Figure 2a). As expected, the immunoglobulin G (IgG) and IgM titers, as evaluated by enzyme-linked immunosorbent assay (ELISA) tests, were identical in both cases.

We also performed a dose–response experiment by injecting B6 mice with 10^9 , 10^{10} , and 10^{11} AAV1-U7 vg. Not surprisingly, AAV1-specific IgM were detected earlier than IgG, peaking at day 7 and then decreasing to a level slightly higher than the basal titer observed in pre-immune sera (Figure 2b). At day 7 after injection, AAV1-specific IgG Abs started to be detected in the sera of mice infused with 10^{11} vg, and rapidly increased thereafter to reach a plateau at week 4 (Figure 2c). Interestingly, mice challenged with 10- or 100-fold less AAV1 particles exhibited the same Ab titer progression, although the final levels were different and were maintained until the end of the experiment 6 months later.

The neutralizing properties of the Abs formed were assayed by transferring serum from injected mice [10^{11} AAV1 vg intramuscularly (IM)] into naïve recipient mice 1 day before the IM AAV1-mSeAP challenge. Figure 2d shows that 600 μ l of immune serum drawn on day 7 (d7) was needed to inhibit 50% of the AAV1-mSeAP transduction, whereas as little as 150 μ l of a d14 immune serum was sufficient to totally block transduction. A partial inhibition was also observed with as little as 30 μ l of a d28 immune serum, certainly because of the higher Ab concentration, and probably also because of affinity maturation of the neutralizing

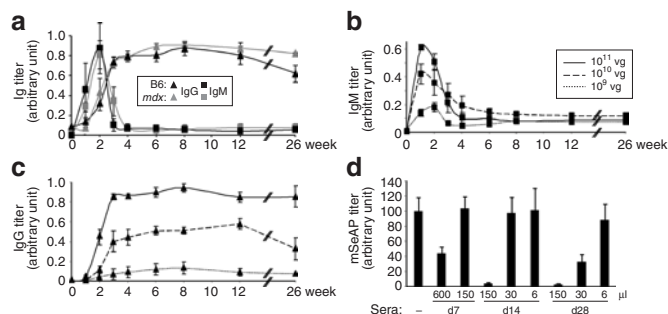


Figure 2 Neutralizing antibodies curtail adeno-associated virus 1 (AAV1) muscle transduction efficiency *in vivo*. **(a)** *Tibialis anterior* (TA) muscles from B6 (black) and *mdx* (grey) mice were injected with 10^{11} AAV1-U7 viral genomes (vg), and the anti-AAV1 immunoglobulin G (IgG) (black or grey triangles) and IgM (black or grey squares) titers were evaluated every week by enzyme-linked immunosorbent assay (ELISA). The data shown represent the mean values from three mice per time point \pm SEM. **(b)** Anti-AAV1 IgM, and **(c)** IgG titers were evaluated after AAV1-U7 injections of 10^{11} , 10^{10} , and 10^9 vg into the TAs of B6 mice. The data shown represent the mean values from four mice per time point \pm SEM. One of two representative experiments is shown. **(d)** From the mice in the experiment described in **b** and **c**, that had received 10^{11} AAV1-U7 vg injections into their TAs, sera were drawn at days 7, 14, and 28 (d7, d14, and d28) after the injections, and these sera were transferred at different doses (600, 150, 30, and 6 μ l) by intravenous injection into naïve mice. One day after serum transfer, these naïve mice were injected in their TAs with 10^{10} vg of AAV1-mSeAP. The data shown represent the mean values of murine secreted alkaline phosphatase (mSeAP) serum levels of three mice per time point \pm SEM. The AAV1-mSeAP expression level of a single infection (lane 1) corresponds to 100% of transduction efficiency. One of two representative experiments is shown.

anti-AAV1 Abs. In this experiment, antigen specificity was verified in terms of the inefficiency of sera from naïve mice or animals immunized twice with AAV2 (data not shown).

Absence of cytotoxic immune response against AAV1 vectors

CTL responses directed against AAV2 capsid epitopes have been recently described in humans^{13,14} and mice.^{15–17} However, there is no direct evidence of an impairment in gene delivery on account of AAV-specific cytotoxic attack *in vivo*. Two factors may contribute toward masking CTL responses directed against the AAV capsid: (i) the very efficient Ab neutralization of AAV particles before their entry into target cells, and (ii) the occurrence of cellular immune responses directed against the transgene itself in some conditions. Using our experimental system, in which mSeAP transgene is not immunogenic *per se*, we monitored CTL responses with the ability to inhibit AAV-based gene transfer in muscle by performing sequential AAV challenges in μ KO mice. In these mice the heavy chain of IgM (μ chain) is invalidated, leading to the absence of mature B-cells.²⁵ Even in the absence of Ab response, we found no evidence of cellular immunity against AAV1 *in vivo*; a second injection of AAV1-mSeAP in μ KO mice was as efficient as the first one using a single-injection protocol (Figure 3a). The absence of cytotoxic activity against AAV1 did not depend upon a delay in CTL induction. This is clear from the fact that a third injection of AAV1-mSeAP 1 month later in μ KO mice was as efficient as a first injection (data not shown). In sharp contrast, we found that when AAV2 vectors were used, the success of gene transfer after secondary challenge was drastically reduced in μ KO mice, showing a 30% reduction in transduction efficiency compared to a single injection (Figure 3b).

By immunostaining whole B6 muscle sections, we observed that each injection provoked a cellular infiltration, probably caused by needle intrusions, but the cellular composition of the infiltrate varied with the number of injections and the AAV serotype (Figure 3c). The infiltrates were composed mainly of CD11b⁺ cells, macrophages, and neutrophils, whose number increased after the second injection. Importantly, we found no CD8⁺ cells in the muscles after one or multiple AAV1 injections, whereas AAV2 transfer was associated with the presence of CD8⁺ cells in the infiltrate of only the secondary injected muscle. CD8⁺ cells were equally recruited after IM infusions of AAV2 in μ KO mice, but never with AAV1 (data not shown).

Taken together, these results demonstrate that the ineffectiveness of AAV1-U7 in the context of rechallenge by multiple infusions is caused only by pre-existing humoral immune response and neutralizing Abs against AAV1, whereas cellular immunity may also play a role in the process of rejection of AAV2 and transduced fibers.

Transient immunomodulation therapy with MR1 and CTLA4/Fc

In order to overcome the humoral immune response to AAV1, we chose to administer an immunomodulation treatment along with the injection of AAV1-U7 in *mdx* mice. The use of classical immunosuppressive treatments such as FK506, mycophenolate mofetil, and cyclosporin have failed to inhibit the formation of

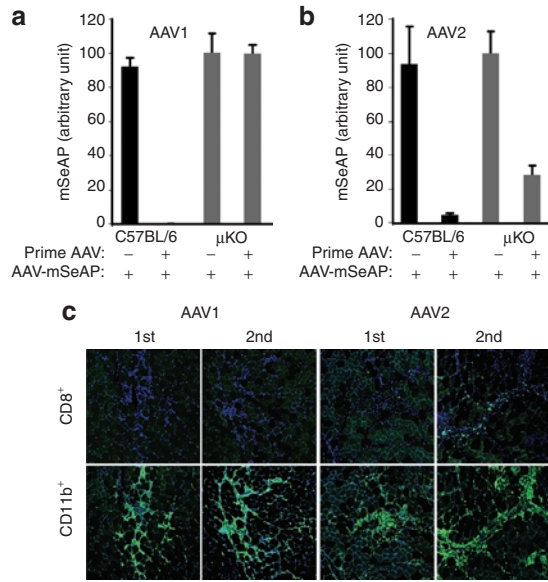


Figure 3 Adeno-associated virus 2 (AAV2)-injected, but not AAV1-injected, muscles present features of cytotoxic attack. (a) B6 (black) and μ KO (grey) mice were either injected in their *tibialis anterioris* (TAs) or not injected with 10^{11} AAV1-U7 viral genomes (vg), and 15 days later they were injected in the contralateral TA with AAV1-mSeAP (10^{10} vg). Serum levels of murine secreted alkaline phosphatase (mSeAP) were measured 15 days after AAV1-mSeAP injection. (b) This experiment was similar to the one described in a except that AAV2 vectors were used. The data shown represent the mean values of mSeAP serum levels of four mice per group \pm SEM. One of two representative experiments is shown. (c) Three days after the last injection, B6 TA sections were immunostained for CD8⁺ and CD11b⁺ cell surface markers. Left panel, results after AAV1 injections; right panel, results after AAV2 injections. Blue, nucleus staining with 4',6-diamidino-2-phenylindole (DAPI); green, CD8⁺ and CD11b⁺ cell surface markers; 1st, after one injection; 2nd, after the second injection. One representative immunostained section of six different muscles is shown for each condition.

anti-AAV1 neutralizing Abs (data not shown). We then explored blocking T/B-cell interactions by interfering with the co-stimulatory pathways B7/CD28 (CTLA4) and CD40L/CD40, using two specific agents, CTLA4/Fc fusion protein and MR1 Abs. We found that transient co-stimulation blockade, performed at day 0 at the time of AAV1-U7 injection and on days 2 and 4, was sufficient to inhibit the formation of anti-AAV1 IgG Abs up to the end of the experiment 6 months later (Figure 4a). The formation of anti-AAV1 IgM Abs was also considerably curtailed by this treatment (Figure 4b). Most importantly, this co-stimulation blockade resulted in the recovery of transduction efficiency after a second AAV1-U7 challenge in the contralateral TA at week 2, as evidenced by the presence of a specific reverse transcriptase polymerase chain reaction (RT-PCR) product from the skipped dystrophin messenger RNA and the detection of rescued dystrophin protein in muscle sections (Figure 5b).

In order to test the feasibility of repeated injections, we performed a third injection in the *gastrocnemius* muscle at day 28. When the immune response in the mice was suppressed during the first AAV1-U7 injection, dystrophin rescue in the third injected muscle was only partial, and anti-AAV1 IgGs appeared 2 weeks later at week 6 (Figure 5b). However, when the mice were immunosuppressed during the second AAV1-U7 injection also,

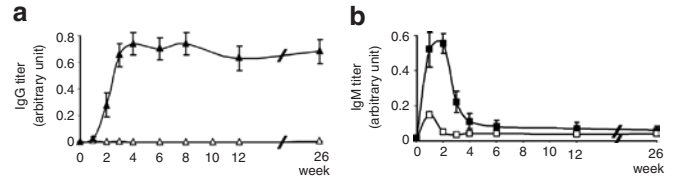


Figure 4 Immunomodulation by co-stimulation blockade inhibits anti-AAV1 immunoglobulin G (IgG) formation. (a) Anti-AAV1 IgG and (b) anti-AAV1 IgM formations in *mdx* mice were evaluated every week by enzyme-linked immunosorbent assay after intramuscular treatment with AAV1-U7 (10^{11} viral genomes) on day 0 (d0). Transient immunomodulation with MR1 and CTLA4/Fc was either carried out intraperitoneally at d0, d2, and d4 (open triangles or open squares) or not carried out (closed triangles or closed squares). The data shown represent the mean values of four mice per time point \pm SEM.

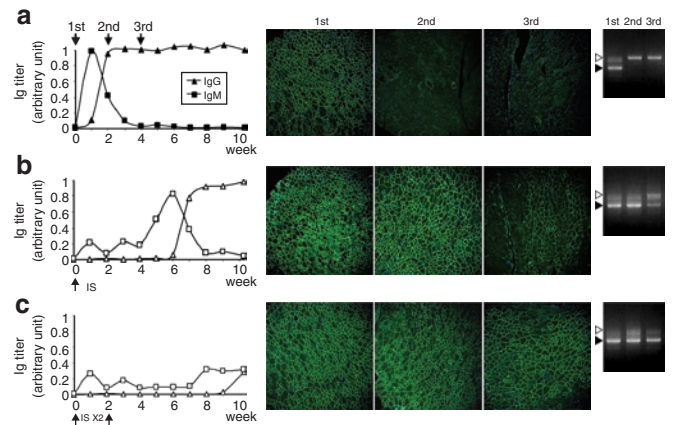


Figure 5 Immunomodulation by co-stimulation blockade restores the efficiency of multiple AAV1-U7 injections. *Tibialis anterioris* (TAs), contralateral TA, and *gastrocnemius* muscles of *mdx* mice were injected with AAV1-U7 (10^{11} viral genomes) at d0 (1st), d14 (2nd) and d28 (3rd). (a) Control without immunosuppression. (b) The mice were immunosuppressed as described for Figure 4, at d0, d2, and d4 (IS, immunosuppression). (c) The mice were immunosuppressed at d0, d2, d4, d14, d16, and d18 (IS X2). Left panels: the mean values of immunoglobulin G (IgG) (closed triangles or open triangles) and IgM (closed squares or open squares) titers from two mice are represented. Middle panels: 6 weeks after the latest injection, dystrophin rescue was monitored by NCL-DYS2 immunostaining on whole transverse sections of muscles. Right panels: RNA samples were analyzed using reverse transcriptase polymerase chain reaction (RT-PCR) for detection of the normal dystrophin (open arrowheads, 901 base pair (bp) fragment) and exon 23-skipped messenger RNAs (closed arrowheads, 688bp). One representative immunostained section, and RT-PCR results from two different muscles is shown for each condition.

the efficiency of gene transfer after the third injection of AAV1-U7 rose to a high level (Figure 5c). Importantly, the level of the RT-PCR product corresponding to skipped dystrophin messenger RNA and the dystrophin rescue evaluated by immunostaining were comparable to those found following the prime injection. In this experiment, IgG titers eventually rose at 2 and 5 weeks after the last AAV1-U7 injection (Figure 5b and c), whereas a single AAV1-U7 injection under co-stimulation blockade did not induce any anti-AAV1 IgG production (Figure 4a). This observation underlines the transient nature of the co-stimulatory blockade. In this study we demonstrate that immunomodulation treatment with CTLA4/Fc and MR1 suppresses the humoral immune

response in a transient manner and should be employed as a co-treatment with each AAV1-U7 injection.

DISCUSSION

Numerous observations have shown that the host immune response to the AAV2 capsid can decrease the efficiency of gene transfer following repeated injections in normal mice, but little is known about AAV1 rejection. In this study, we precisely characterized the nature of the immune responses to AAV1 in dystrophic mice, and assessed the efficiency of immunosuppressive treatments in minimizing the risk of immune rejection of this therapeutic vector. This is particularly relevant for the restoration of muscle function in human DMD patients, as they will require repetitive AAV infusions to target several muscles territories. We demonstrate here that an IM injection of AAV1-U7 induces only a humoral immune response directed against the AAV vector itself, precluding the success of subsequent AAV1 infusions if administered more than 3 days after the primary injection (Figure 1). Furthermore, we found that this adverse immune response to AAV1 vectors was essentially due to the generation of AAV1 neutralizing Abs, as shown by a novel AAV neutralization assay that we performed *in vivo* by transferring immune sera into naïve mice (Figure 2d). In agreement with this finding, immunoglobulin-deficient μ KO mice were readily transduced with multiple rounds of AAV1 vectors (Figure 3a). Interestingly, while the AAV1 serotype induced no adverse immune response for secondary injections in immunoglobulin-deficient mice, the AAV2 serotype induced an immune response, which lowered the efficiency of gene transfer during secondary injections in these mice (Figure 3b). This was presumably caused by cellular immune responses, in view of the finding that CD8⁺ T cells were present in muscle tissue injected with AAV2 in the C57BL/6 genetic background (Figure 3c). These results strongly suggest that, despite high homology between the capsid amino acids of AAV1 and AAV2 (more than 80%), vector particles exhibit a great difference in their ability to elicit cytotoxic T-cell responses. We cannot yet exclude the possibility that cytotoxic responses to AAV2 would in fact be directed against a factor which co-purifies with AAV2. However, as has been recently elucidated in mice and in nonhuman primates, the AAV2 capsid domain contains a heparan sulfate proteoglycan RXXR-binding motif on the VP3 protein, which directs T-cell activation.¹⁷ This RXXR heparan sulfate proteoglycan-binding motif potentiates AAV2 uptake in human dendritic cells and can activate capsid-specific T cells in mice without really affecting gene transfer efficiency after the first injection.¹⁷ It is noteworthy that AAV1 capsid proteins do not carry this RXXR motif that is present on AAV2 (ref. 18). This can certainly explain the lack of CD8⁺ T-cell infiltration found after secondary muscle injections with AAV1, as well as the residual adverse immune response to AAV2 found in μ KO mice lacking the humoral arm of the humoral response. A recent study also indicated that AAV2 and AAV6 capsid proteins can elicit cellular immune responses when injected into the skeletal muscles of random-bred dogs, but not in inbred mice.²⁶ AAV6 is very similar to the AAV1 serotype, but differs by six amino acids in the capsid proteins and does not harbor the RXXR motif.²⁷ Interestingly, in our experiments with golden retriever muscular dystrophy dogs, no cellular infiltrates were ever observed in AAV1-U7 injected

muscles,² and it is likely that motifs other than RXXR might trigger a cellular response against AAVs. As things stand, AAV1 lacks these motifs, at least in the murine and canine contexts.

We found here that the humoral immune response to AAV1 is very potent and prevents the success of a second challenge as early as 3 days after the first IM injection (Figure 1). AAV1 induced an early IgM peak during the first week, while IgG concentration increased and reached a plateau at 3–4 weeks (Figure 2). In order to assess the importance of AAV1-specific Ig production for repetitive AAV1 gene delivery, we set up a novel *in vivo* neutralization assay taking into account the neutralization and opsonization effects of AAV1 viral capsids, given that both neutralizing and non-neutralizing Abs stimulate phagocytosis and clearance of viral particles.^{28,29} This test addresses an important concern, because the *in vivo* neutralization capacity of Abs is often underestimated by the classical *in vitro* transduction neutralization assay.³⁰ In our study, effective *in vivo* neutralization occurred within a few days (Figure 1), and the strength of this immune response increased thereafter (Figure 2d), probably as a result of both quantitative (Ab concentration) and qualitative (isotype switching and affinity maturation) effects. While 600 μ l of day 7 serum from AAV1-U7-injected mice is needed to partially inhibit virus entry, as little as 30 μ l of a day 28 serum is sufficient to reduce the transduction efficiency to two-third of its original value.

Various approaches have been devised to achieve infusion of AAV vectors into hosts with pre-existing neutralizing Abs.³¹ In particular, in a recent report, the error-prone PCR and DNA-shuffling strategies have been used for generating AAV capsid variants which evade the effects of neutralizing Abs.^{32,33} Injections of noncross-reactive serotypes has also been used for achieving effective multiple challenges in mouse muscles. Along these lines, our research group as well as others have shown that no significant cross-neutralization occurs after intramuscular delivery between AAV1, AAV2, and AAV5.^{22,23,34} However, this strategy raises several difficulties for clinical application, because several clinical-grade vector batches (one per serotype) have to be produced and validated. Moreover, the therapeutic benefit would be reduced by the limited tropism for muscle of serotypes such as AAV2 and AAV5, and one could not exclude the possibility of unknown cross-reactivities between these AAV serotypes in the human system. It is also important to note that, unlike mice, humans are natural hosts for AAV, and that the nature of the immune response probably depends on the initial adenovirus co-infection required to promote productive AAV infection.

From the results of our study and those of others, it is evident that, even though AAV1 vectors do not transduce dendritic cells efficiently and may not face the problem of a pre-existing humoral immunity in humans,¹⁷ they would nevertheless induce a strong humoral immune response, thereby precluding the success of secondary injections into muscles. In order to eliminate this adverse immunity, we explored methods to block interactions between T cells, antigen-presenting cells, and B-cells by interfering with the co-stimulatory pathways B7/CD28 (CTLA4) and CD40L/CD40, using two specific agents, CTLA4/Fc and MR1, which are currently being evaluated in human clinical trials. We tested the efficiency of this strategy for extensive IM delivery of AAV1-U7 vector in *mdx* mice, and assessed the results by monitoring dystrophin rescue

directly in large muscle territories. This transient inhibition of co-stimulatory molecules, performed along with each AAV1-U7 injection, was sufficient to block the immune response to AAV1, as evidenced by the fact that no Abs were detected up to the end of the experiment 6 months later (Figure 4), and that dystrophin rescue was allowed, using up to three injections administered into multiple dystrophic muscles (Figure 5).

CTLA4/Fc has been used successfully used as an immunosuppressive regimen in animal models of autoimmunity³⁵ and transplantation³⁶ and has been approved by the Food and Drug Administration for clinical use. The murine CTLA4/Fc and the anti-mouse CD40L monoclonal antibody (mAb) (MR1) are known to act in synergy³⁷ and to facilitate AAV-mediated transgene expression after both primary and secondary vector injection into lungs³⁸ and muscles.¹² Clinical trials that tested the efficacy and safety of anti-CD40L have yielded controversial results relating to increased risk of thrombosis in patients.^{39,40} In our study, however, in contrast to experiments dealing with organ or transgene rejection, we wished to blunt the responses to the AAV capsid antigen load present only at the moment of the IM infusion. The known toxicity of the immunomodulating agents could therefore be offset by the short duration of the treatment required. However, as shown in Figure 5b, full immunosuppression lasts no more than 4 weeks, and the third injection performed at 28 days after treatment was only partially effective. Therefore this approach is only transiently efficacious and implies that vector re-administration has to take place within a narrow window of time between injections.

It is important to note also that dystrophic muscles have persistently elevated $\text{I}\kappa\text{B}$ kinase/NF- κB signaling in immune cells and muscle fibers, and this was recently shown to promote inflammation and limit regeneration of muscle progenitor cells.⁴¹ Our data demonstrate that the inhibition of co-stimulatory molecules with CTLA4/Fc fusion protein and anti-CD40 Abs blocked the initiation of humoral response toward AAV1-U7 vector particles injected into *mdx* mouse muscle tissues that are highly susceptible to inflammation. Overall, our results indicate for the first time that a realistic therapeutic strategy is now feasible for treatment of DMD using repetitive injections of AAV1-U7 viral vectors.

MATERIALS AND METHODS

Mice and immunosuppressive treatment. All animal procedures were performed according to an institution-approved protocol and under appropriate biological containment. Six-week-old *mdx*, C57BL/6 (Charles River Laboratories, L'Arbresle, France) and μKO mice²⁵ (CDTA, Orléans, France) were injected with 50 μl phosphate-buffered saline containing AAV vectors into the TA and *gastrocnemius* muscles. Immunosuppressed mice received 6 mg/kg/day MR1 (ref. 42) and 5 mg/kg/day murine CTLA4/Fc (Interchim, Montluçon, France) intraperitoneally at d0, d2, and d4, where d0 represents the day of gene transfer. For other immunosuppressive therapies, mice were injected intraperitoneally during 15 days, with: FK506 2.5 mg/kg/day (Sigma Aldrich), or mycophenolate mofetil 40 mg/kg/day (Roche), or mycophenolate mofetil 40 mg/kg/day plus prednisone 0.25 mg/kg/day (Sigma Aldrich, Lyon, France) or cyclosporin 5 mg/kg/day (Sigma Aldrich, Lyon, France). Blood samples were obtained by retro-orbital puncture of anesthetized animals. After the mice were killed, the muscles were collected, snap-frozen in liquid nitrogen-cooled isopentane, and stored at -80°C .

Recombinant AAV vector production. AAV2/1 and AAV2/2 vectors were generated using a three-plasmid transfection protocol as described²³ with pGG2-CMV-muSeAP for AAV1-mSeAP vector production,²³ pAAV-C5-12-mSeAP for AAV2-mSeAP⁴³ and pAAV(U7smOPT-SD23/BP22) for AAV1-U7 and AAV2-U7.¹

In vivo transduction efficiency analysis. Determination of alkaline phosphatase concentration in sera and histochemical detection of transduced muscle sections were performed as described.²³ Dystrophin RNA exon skipping was analyzed by RT-PCR and dystrophin rescue by immunohistochemistry, using protocols described previously.¹ Briefly, nested RT-PCR was carried out with 200 ng of TRIzol (Life Technologies) isolated total RNA using Access RT-PCR system (Promega). The first reaction was performed with Ex20ext and Ex26ext primers and the second with Ex20int and Ex26int.

Immunofluorescence analysis. A series of 8 μm transverse sections cut at 200 μm intervals over the muscle length were examined by immunohistochemistry for CD8⁺ (CT-CD8a mAb; Caltag Laboratories) and CD11b⁺ (M1/70 mAb; BD Pharmingen) cell surface markers. mAb were detected using biotinylated mAb followed by streptavidin-alexa 488 (Molecular probes). Mounted sections were analyzed using confocal laser microscopy (Leica).

Detection of anti-AAV1 Abs. Serum Ab titers against AAV1 capsid were determined by ELISA as previously described by others⁴⁴ using 10^9 vg AAV1-mSeAP for plate coating, and BD Assay Diluent (BD Biosciences) as blocking reagent. Finally, plates were revealed with horseradish peroxidase-conjugated goat anti-mouse IgG and IgM (Southern Biotech) and TMB Substrate reagent Set (BD Biosciences).

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