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Effect of rapamycin on immunity induced by vector-mediated dystrophin expression in *mdx* skeletal muscle

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Duchenne muscular dystrophy (DMD) is caused by mutations in the dystrophin gene. Therapeutic gene replacement of a dystrophin cDNA into dystrophic muscle can provide functional dystrophin protein to the tissue. However, vector-mediated gene transfer is limited by anti-vector and anti-transgene host immunity that causes rejection of the therapeutic protein. We hypothesized that rapamycin (RAPA) would diminish immunity due to vector-delivered recombinant dystrophin in the adult *mdx* mouse model for DMD. To test this hypothesis, we injected limb muscle of *mdx* mice with RAPA-containing, poly-lactic-co-glycolic acid (PLGA) microparticles prior to dystrophin gene transfer and analyzed treated tissue after 6 weeks. RAPA decreased host immunity against vector-mediated dystrophin protein, as demonstrated by decreased cellular infiltrates and decreased anti-dystrophin antibody production. The interpretation of the effect of RAPA on recombinant dystrophin expression was complex because of an effect of PLGA microparticles.

D uchenne muscular dystrophy (DMD) is an X-linked, fatal disorder affecting 1 in 3600–6000 male births worldwide[1–3]. This progressive disease is due to mutations in the dystrophin gene that result in absence of expression of a functional dystrophin protein in skeletal and cardiac muscles. Dystrophin is an essential muscle protein that binds filamentous actin and the membrane-bound dystroglycan complex providing a structural link from the intracellular cytoskeleton to the extracellular basal lamina of muscle fibers[4–6]. Absence of dystrophin protein alters muscle fiber membrane structure and function, causing membrane damage during muscle contraction and disruption of signaling across the membrane. The *mdx* mouse is a disease model of DMD, in which, a mutation in the dystrophin gene causing absence of dystrophin protein in muscle fibers leads to necrosis and inflammation in muscle tissue[7].

Gene replacement has potential utility in the treatment of single-gene disorders, such as DMD. Since the dystrophin gene is very large comprising 11 kb of coding sequence, transfer of the full-length dystrophin cDNA into muscles of dystrophic mice was only possible due to the development of high capacity adenoviral (HC-Ad) vectors, which retain no viral genes and thus have a large capacity for an inserted DNA expression cassette[8–11]. The lack of viral genes in the HC-Ad vector leads to a lower induction of anti-vector immunity than prior generation Ad vectors and has also been shown to facilitate prolonged dystrophin protein expression in the *mdx* mouse[9].

The host immune system, however, is a major obstacle to successful transfer of a full-length dystrophin cDNA to dystrophin-deficient muscles. Therefore, immune suppression in a dystrophic host may prevent host immunity against recombinant dystrophin protein and vector particles. Immunosuppression can be delivered either systemically or locally.

For the purposes of testing our hypothesis, we chose to use local immunosuppression, delivered directly to skeletal muscle tissue using injectable, biocompatible, and degradable microparticles (beads). A significant advantage of this approach is the slow release of the drug administered locally, thus avoiding the need for multiple injections. We previously reported rapamycin (RAPA) drug delivery from beads injected locally in limb muscle of *mdx* mice[12]. We showed that bead-delivered RAPA significantly lowered inflammation in dystrophic limb muscle of adult *mdx* mice. Effects of RAPA have also been investigated for therapy of autoimmunity and to facilitate

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organ or tissue transplantation[13,14]. We hypothesized that bead-delivered RAPA would suppress anti-dystrophin immunity in dystrophic *mdx* muscle tissue treated with dystrophin vector delivery, and thus provide proof-of-principle that RAPA could promote the success of dystrophin gene transfer to skeletal muscle.

To test this hypothesis, we delivered RAPA-containing beads to tibialis anterior (TA) muscles of the adult *mdx* mouse together with a direct intramuscular injection of an HC-Ad vector carrying full-length murine dystrophin driven by a muscle-specific promoter. We studied transgene expression, T lymphocyte infiltration and the humoral immune response to the transgene product. The beads used in these studies have an average diameter of about 15 μm and thus remain at the site of delivery in muscle tissue and are not easily picked up by circulating dendritic cells, thus achieving a local treatment.

Results

RAPA treatment reduced anti-dystrophin antibody production following vector-mediated dystrophin expression in mdx muscle. In order to examine if injection of RAPA-containing beads into *mdx* muscle could suppress humoral host immunity against vector-mediated dystrophin expression in the treated tissue, we examined the sera from RAPA bead-treated, vector-injected mice for production of anti-dystrophin antibodies. We compared the sera from RAPA bead-treated mice to sera from mice that had received blank bead injections prior to an intramuscular dystrophin vector injection in the TA muscles. For this analysis, 6 weeks following RAPA bead or blank bead administration with dystrophin vector delivery, anti-dystrophin antibody production was analyzed in mouse serum, and vector-mediated dystrophin expression was detected in target muscle tissue.

While there was variability in the humoral response against dystrophin protein, there were a greater proportion of the mice that received RAPA bead treatment prior to dystrophin vector transfer that produced lower levels of anti-dystrophin antibodies, when compared to the blank bead-injected mice (Fig. 1). Five out of 6 RAPA bead-treated mice showed no more than a mild humoral response to dystrophin protein as compared to only 2 out of 5 blank bead-treated mice. Although the variability between individual mice tempers the conclusion, this observation suggested that local RAPA treatment reduced anti-dystrophin humoral response compared to blank bead-injected mice, but did not lead to a complete elimination of host humoral immune response against vector-mediated dystrophin protein expression in *mdx* mice.

RAPA treatment reduced infiltration of effector T cells, but not regulatory T cells in mdx muscle. To examine the effect of RAPA on T cell infiltration following vector-mediated dystrophin expression, CD4$^+$, CD8$^+$, and Foxp3$^+$ cells were quantitated in TA muscles of *mdx* mice treated with RAPA or blank beads prior to dystrophin vector injection. T cell infiltration was analyzed by immunohistochemistry (Fig. 2C) and average cell numbers per TA cross-section were compared. CD4$^+$ T cells were significantly lower in RAPA bead-treated, vector-injected *mdx* TA muscles compared to both

blank bead-treated, vector-injected TA muscles (p=0.014), and mice that received only dystrophin vector (p=0.007) (Fig. 2A). CD8$^+$ T cells were also at lower levels in RAPA bead-treated, vector-injected *mdx* muscles compared to blank bead-treated, vector-injected muscles, but the difference was not significant (p=0.13). Interestingly, however, the level of Foxp3$^+$-expressing regulatory T (Treg) cells was not lower in RAPA bead-treated muscles compared to blank bead-treated muscles (Fig. 2A). The effect of RAPA bead treatment on the ratios of Treg cells to effector CD4$^+$ or CD8$^+$ cells suggests that Treg cells down-regulated effector T cell proliferation in RAPA bead-treated, vector-injected TA muscles (Fig. 2B).

Both RAPA bead and blank bead treatment was associated with a higher level of vector-mediated dystrophin expression in mdx muscles. Immunohistological detection of dystrophin protein in muscle cross-sections revealed vector-mediated dystrophin expression in RAPA bead-treated muscles that was significantly higher than dystrophin expression in muscles injected with the vector without any prior treatment and the background level of dystrophin expression in revertant fibers in untreated age-matched muscles (Fig. 3). Interestingly, mice injected with blank beads and dystrophin vector showed a similar level of dystrophin expression (p = 0.98) when compared to RAPA bead-treated, dystrophin vector-injected mice, at 6 weeks following treatment (Fig. 3).

Discussion

Evasion of the host-anti-dystrophin immune response is required for successful therapeutic gene replacement in DMD patients, and has been studied in animal models of DMD including the *mdx* mouse[15–19]. Therefore, down-regulation of the host immune system prior to and during vector transfer and vector-mediated gene expression is important to the success of therapeutic dystrophin gene transduction of muscles of the dystrophic host.

The immunosuppressive drug, RAPA, has demonstrated suppression of immunity in multiple clinical applications[20,21]. Mechanisms of action of RAPA for immune suppression include Treg expansion, inhibition of effector T cell activation and proliferation, and prevention of inflammatory cytokine production[22–24]. In a wide range of transplantation studies, including renal and bone marrow transplantation, the use of RAPA to reduce or eliminate both graft-versus-host and host-versus-graft responses has been successful[23,25–28]. RAPA has also been used in transplantation of myoblasts in *mdx* mice[29]. We previously showed that RAPA ameliorates the dystrophic phenotype in skeletal muscle of adult *mdx* mice[12]. Therefore, we hypothesized that the effects of RAPA to decrease immunity in skeletal muscle of the *mdx* mouse could benefit vector-mediated dystrophin gene replacement.

We and others previously showed that vector-mediated dystrophin expression in *mdx* muscles caused effector T cell infiltration at levels significantly higher than untreated *mdx* muscles[15,30]. In agreement with these findings, we show here that treatment of mice with blank beads prior to dystrophin vector transfer, results in a higher level of CD4$^+$ and CD8$^+$ T cell infiltrates in treated muscle tissue and a strong humoral response to dystrophin. RAPA bead treatment

Figure 1 | Anti-dystrophin humoral response in *mdx* mice treated with rapamycin (RAPA) bead or blank bead injections prior to dystrophin vector injection. At 6 weeks of age HC-AdmDYS vector was injected into tibialis anterior muscles of RAPA bead- (RAPA+DYS, n=6) or blank bead-injected (Blank+DYS, n=5) or uninjected (only DYS, n=3) *mdx* mice. Muscle tissue was collected 6 weeks post-treatment. Sera were analyzed for anti-dystrophin antibody production. A monoclonal anti-dystrophin antibody (Dys-2) was used as a positive immunoblotting control. Negative controls were sera from untreated age-matched *mdx* mouse and a sample with no serum incubation (secondary antibody only).
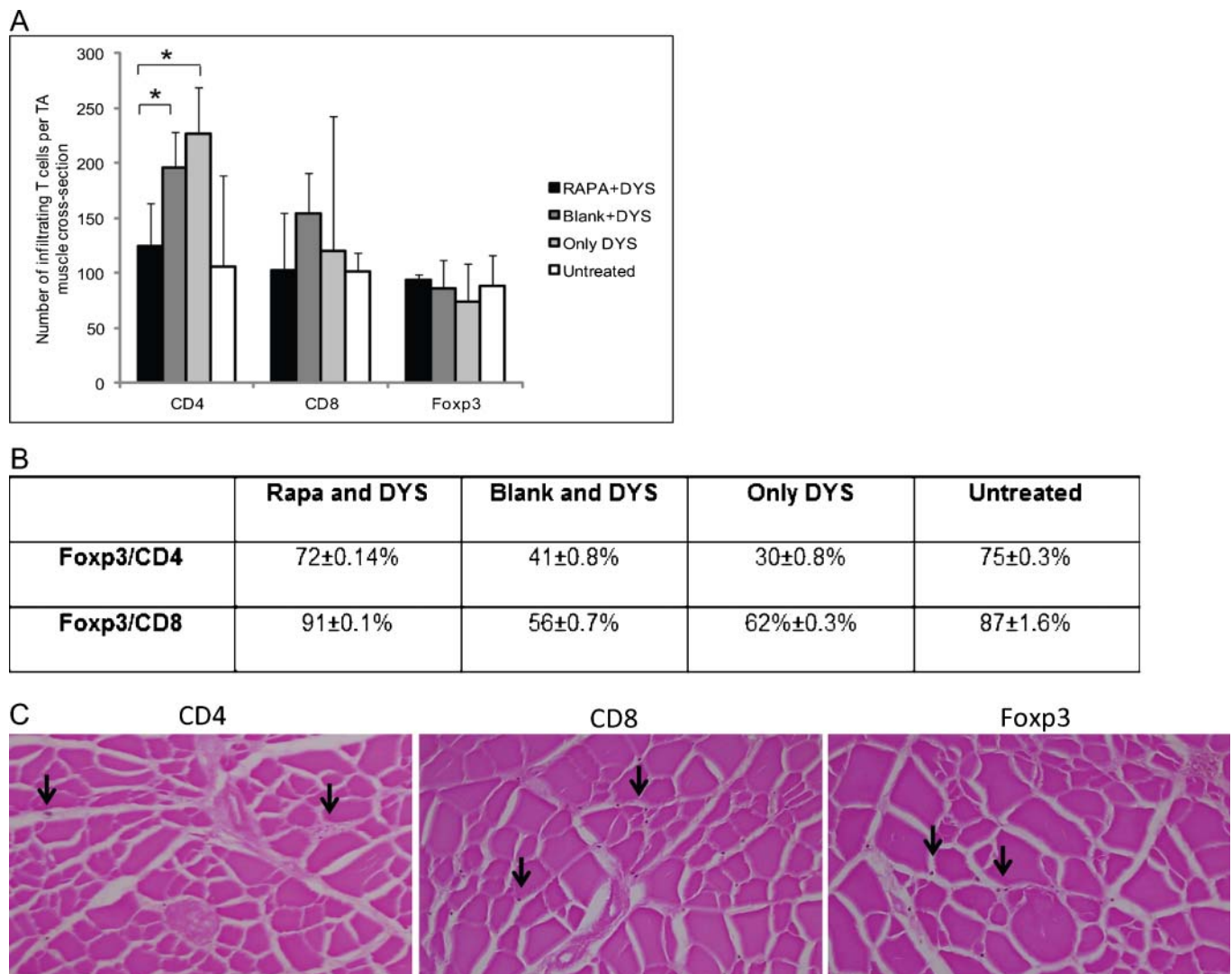
Figure 2 | T cell infiltration in rapamycin (RAPA) bead- or blank bead-treated, dystrophin vector-injected _mdx_ tibialis anterior (TA) muscles. Six weeks following treatment of adult _mdx_ mice with RAPA beads and dystrophin vector (RAPA+DYS, n=4), blank beads and dystrophin vector (Blank+DYS, n=3), no beads and dystrophin vector (Only DYS, n=3), or untreated controls (n=3), TA muscles were analyzed for T cell infiltration. CD4^{+}, CD8^{+}, and Foxp3^{+} T cells were detected by immunohistochemistry (IHC) and averaged per TA cross-section. (A) shows the total number of each type of T cell per TA cross-section and (B) shows the percentages of Foxp3^{+} cells as a function of total effector CD4^{+} and CD8^{+} cells. (C) shows representative IHC images of T cell infiltration in RAPA bead-treated mice. * indicates p value < 0.05.

prior to dystrophin vector transfer reduces infiltration of effector T cells to levels comparable to non-transduced _mdx_ muscle, indicating that RAPA decreases host cellular immunity following vector-mediated dystrophin expression. This down-regulation of immunity to vector-mediated dystrophin expression may be mediated by Treg cells, as the ratios of Treg cells to both CD4^{+} and CD8^{+} effector T cell populations increased in RAPA-treated, vector-injected TA muscles compared to the mice that received either only the vector, or blank beads and vector. The effect of RAPA on selective survival of Treg cells, which can then lead to the increase of their ratio over effector CD4 and CD8 T cells shown in our study, is in agreement with previous findings where RAPA induced preferential expansion of Treg cells in other studied tissues[21,22,31,32]. Furthermore, this study demonstrates RAPA bead-mediated suppression of humoral immunity specific to recombinant murine dystrophin expression, although variability between mice and small sample size limit the strength of conclusions that can be drawn from this observation. Although not specifically studied in our experiments, RAPA treatment would also be expected to reduce humoral and cellular

immunity to vector capsid proteins observed in other studies[33]. Since the vector used in these studies is a high-capacity Ad vector, capsid proteins are only introduced by the initial vector dose[30], and possibly to a very low continuing level by small amounts of helper virus contained within the high-capacity Ad vector preparation.

PLGA beads are known to degrade into acidic components (pH≈6.0), which can lead to a lowering of pH in the target tissue[34,35]. In fact, the pH of bead-injected tissue is usually between pH 6.0 – 7.0[35], but can go down to as low as pH 5.0 if the environment is not buffered well. Previous studies have indicated that lowering the pH of a tissue environment may promote gene expression, as one study showed 2–14 fold increase in protein synthesis when pH was lowered from 7.0 to 5.0 in bacterial systems[36]. To our knowledge there is no published literature addressing a similar effect of acidity on protein expression in mammalian systems. Our data showed that administration of RAPA beads or blank beads in conjunction with dystrophin gene delivery to muscle yielded comparable levels of vector-mediated dystrophin expression observed 6 weeks after gene transfer. Over the same time period, vector-mediated dystrophin
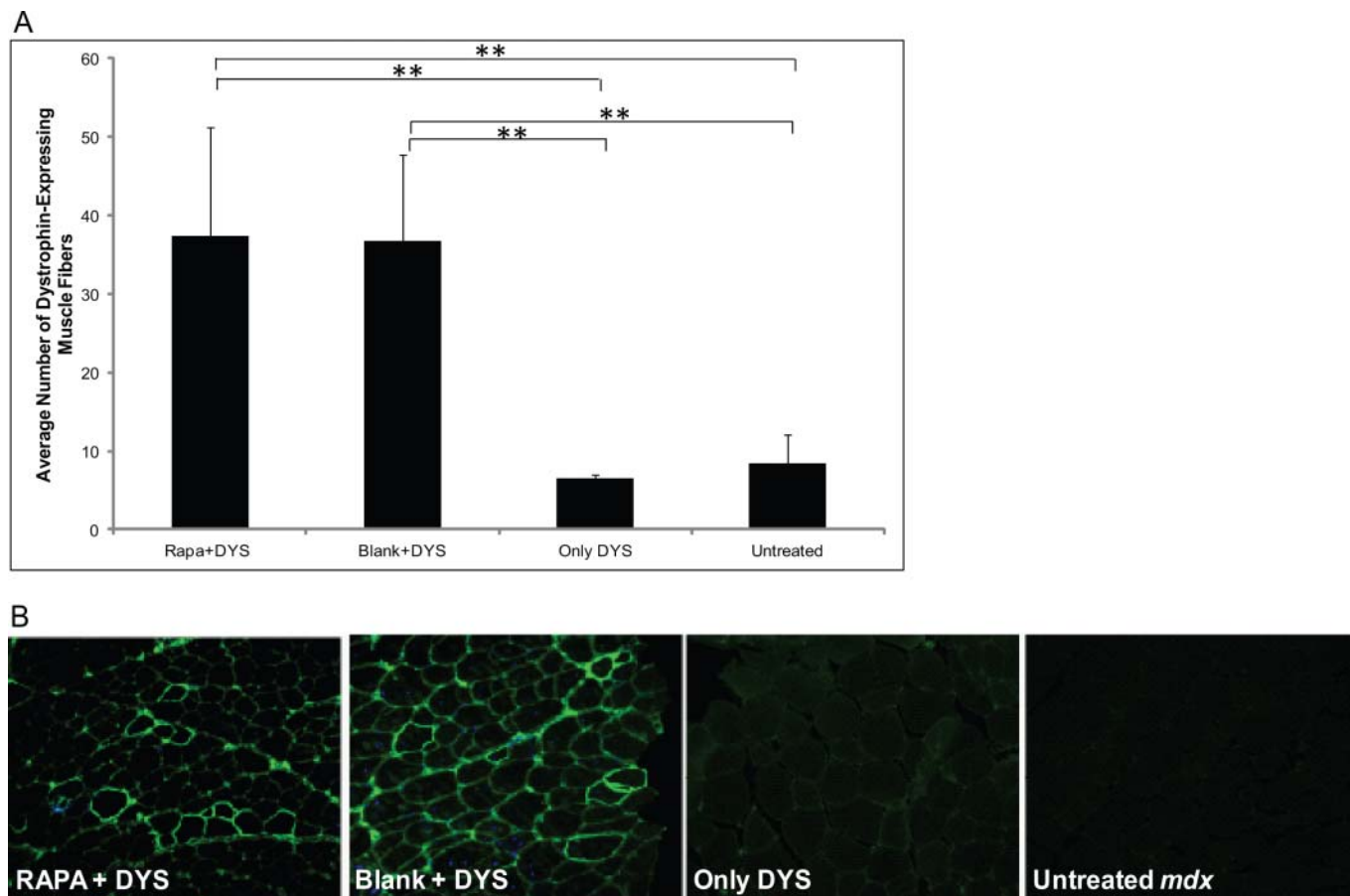
Figure 3 | Vector-mediated dystrophin protein expression in rapamycin (RAPA) bead- or blank bead-treated *mdx* tibialis anterior (TA) muscles. At six weeks following treatment of adult *mdx* mice with RAPA beads and dystrophin vector (RAPA+DYS, n=6), blank beads and dystrophin vector (Blank+DYS, n=5), no beads and dystrophin vector (Only DYS n=3), and untreated (n=3) *mdx*, TA muscles were analyzed for dystrophin protein expression. Dystrophin-expressing fibers were counted on cross-sections of TA muscles throughout the length of each TA muscle and were averaged per section (A). Detection of dystrophin-expressing fibers was by immunohistochemistry using a monoclonal anti-dystrophin primary antibody (Dys-2) (B). ** indicates p value < 0.005.

expression was lost in control animals that received dystrophin gene transfer without either type of bead. A possible explanation for the observation that vector-mediated dystrophin expression is significantly higher in both bead-treated groups compared to the group that only received vector injection is the effect of the acidic nature of the beads that may promote successful vector-mediated gene expression.

Despite the demonstration that immunosuppression induced by RAPA may decrease anti-dystrophin antibody production and cellular immunity in dystrophin vector-injected mice, RAPA bead-treated, vector-injected *mdx* muscle did not show a higher level of vector-mediated dystrophin expression than blank bead-treated, vector-injected *mdx* muscle. One factor leading to this finding could be the binding of RAPA to the mammalian target of RAPA (mTOR), which is a major negative regulator of cell growth and protein synthesis in response to energy and nutrients[37–39].

In summary, our studies demonstrate that local administration to skeletal muscle of RAPA using PLGA beads may reduce both humoral and cell-mediated immunity caused by dystrophin gene therapy. However, the finding of increased dystrophin expression at 6 weeks after gene transfer with either blank or RAPA beads suggests that a component of the beads themselves, such as a local acidic environment, benefits recombinant gene expression. The observation that RAPA does not further increase recombinant gene expression above the level of blank beads suggests that RAPA inhibits expression of the transgene, possibly through its known mechanism to decrease protein synthesis in muscle.

Methods

Mice: Wild type (C57BL/10J) and *mdx* (C57BL/10ScSnDmd*mdx*/J) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in a pathogen-free facility and were fed with autoclaved food and autoclaved acidic water (pH 2.5 after adding HCl). The care and use of mice for this study was approved by the University of Pittsburgh IACUC.

RAPA-containing PLGA bead preparation: RAPA beads were produced as described[40]. In brief, RAPA beads were produced using the single emulsion-evaporation technique that involved forming an emulsion of an organic solution containing RAPA and PLGA in a bulk aqueous solution, through high speed homogenization at 3000 rpm (Silverson L4RT-A, Chesham Bucks, UK). Following evaporation of the organic solvent (dichloromethane), beads formed in the aqueous solution were freeze-dried prior to use in experiments.

Intramuscular bead injection: Six-week-old *mdx* mice were injected intramuscularly with RAPA-containing beads (RAPA beads) in sterile PBS (30 μl per TA). Control mice either received empty beads (blank beads) or were left untreated.

Dystrophin HC-Ad vector: The HC-Ad vector contains a full-length murine dystrophin cDNA under the control of the muscle creatine kinase (MCK) promoter, the left and right viral inverted terminal repeats (ITRs) of adenovirus serotype 5 and hypoxanthine-guanine phosphoribosyl transferase (HPRT) 'stuffer' DNA. The construction of the HC-AdmDYS vector has been described previously[11,41].

Intramuscular vector injections: At 48 hours post-injection of RAPA beads and blank beads, the mice received inhalational anesthesia with isoflurane and were injected with $1.0-2.0 \times 10^{10}$ genome copies of HC-AdmDYS vector intramuscularly in the TA muscle bilaterally. Each muscle was injected with a volume of 20 μl in PBS, using a 28 G needle (B–D; Franklin Lakes, NJ).

Total muscle protein extract (TMPE) preparation: Freshly isolated mouse muscle was cut in small pieces in TEES buffer (50 mM Tris-HCl pH 8.0, 5 mM EGTA pH 7.4, 5 mM EDTA pH 8.0, 5% SDS) and incubated on ice for 45 minutes. Samples were then sonicated briefly and centrifuged at 14,000 rpm for 20 minutes at 4°C. The supernatant was collected and stored at −80°C.

Gel electrophoresis and Western blotting: Following standard protocols, the membranes with immobilized murine dystrophin protein used for the assay were generated from wild-type C57BL/10J (B10) TMPE electrophoresed on SDS-PAGE. In brief, TMPE from B10 mice were run on a 5% Acrylamide gel (Bio-Rad; Hercules, CA) for 3 hours at 110 V. Protein samples were transferred from the gel to nitro-cellulose membrane (GE Healthcare; Piscataway, NJ) for 1.5 hours at 110 V at 4°C. The membrane was blocked in 5% milk/1% sheep serum/TBST (10 mM Tris pH 8.0, 0.15 M NaCl, 0.5 mM Tween-20) overnight at 4°C. The membrane was then cut in pieces with a dystrophin protein band in each piece. The pieces of membrane were then each incubated with a diluted serum sample (1 : 300) in TBST for 1.5 hours, then with HRP-conjugated sheep anti-mouse IgG (GE Healthcare) diluted in TBST for 45 minutes at room temperature. ECL detection reagent (GE Healthcare) was used to detect the chemiluminescent signal and Kodak film was used to visualize the signal. The initial exposure time was 15–30 minutes and to confirm the absence of a band, an additional film was exposed to the membrane overnight.

Muscle tissue processing for histology: Freshly dissected muscle was incubated in 2% paraformaldehyde/PBS on ice for 2 hours, then transferred into 30% sucrose over-night at 4°C. The next day the muscle tissue was snap-frozen in 2-methylbutane cooled with dry ice and stored at −80°C.

Immunohistochemistry of inflammatory cells: Immunohistochemistry for detection of inflammatory cells in muscle tissue was performed as we have previously shown[12,30]. In brief, 10μm cryo-sections of muscle samples were rehydrated in PBS, blocked in peroxidase blocking reagent (DAKO Cytomation; Carpinteria, CA) for 5 minutes, and then blocked in 10% goat serum/PBS for 1 hour at room temperature. The primary antibody incubation using rat anti-CD4 and rat anti-Foxp3 (ebiosciences; San Diego, CA) and rat anti-CD8 (Pharmingen; San Jose, CA) purified antibodies diluted in 10% goat serum/PBS were done for 1.5 hours at room temperature. Sections were incubated for 1 hour with secondary biotinylated goat anti-rat IgG (Pharmingen) diluted in DAKO antibody diluent (DAKO Cytomation). Sections were incubated in ABC Vectastain avidin-HRP detection solution (Vector Laboratories; Burlingame, CA) for 30 minutes at room temperature and DAB per-oxidase substrate solution (Vector Laboratories) for 4 minutes. Eosin counterstaining was performed for visualization of muscle fibers. To analyze infiltrating cells in each group, the total number of cells per cross-section of vector-injected TA muscles was counted for 3–5 cryo-sections distributed through the length of the muscle. From this data, the average number of cells per cross-section was calculated for each experimental mouse.

Immuno-fluorescence detection of dystrophin: 10 μm cryo-sections of muscle samples were rehydrated with PBS, blocked first with avidin and biotin block (Vector Laboratories), and then with mouse IgG block (Vector Laboratories), according to the manufacturer's instructions. Incubation with primary anti-DYS (Dys-2; Novocastra Laboratories, UK) was done for 3 hours. Sections were then incubated with bioti-nylated goat anti-mouse IgG (Pharmingen) and tertiary FITC-conjugated donkey anti-goat IgG (Jackson ImmunoResearch Laboratories, Inc.; West Grove, PA).

Statistical Analysis: For all studies, statistical analysis was performed by one-way ANOVA, in which a treatment group was compared to other groups in the same category. Values were presented as the mean with standard deviation (SD). In all experiments, P values <0.05 were considered significant.

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Author contributions

SE designed the study, performed experiments, analyzed data, and wrote the manuscript. SJ prepared reagents, analyzed data and edited the manuscript. SRL supervised the preparation of reagents, analyzed data and edited the manuscript. PRC designed the study, supervised experiments, analyzed data and edited the manuscript.

Additional information

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