Assessment of Immunomodulatory Regimens that Allow Systemic Redosing of an AAV-CRISPR/Cas9 Therapy for Duchenne Muscular Dystrophy

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INTRODUCTION

- **Duchenne muscular dystrophy (DMD)** is a degenerative muscle disease caused by mutations in the *DMD* gene resulting in a lack of dystrophin protein.

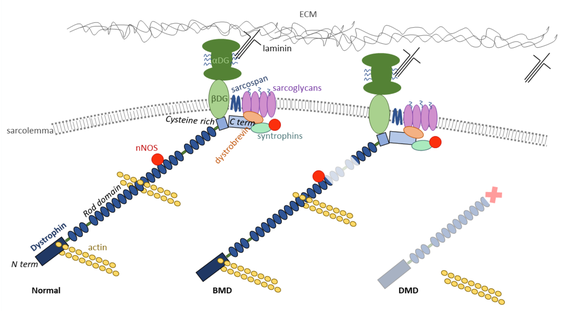

Figure 1: Dystrophin in skeletal muscle. Dystrophin links actin to the extracellular matrix via the dystrophin glycoprotein complex (DGC) and keeps the complex associated with the membrane. DMD causes complete lack of dystrophin whereas BMD in-frame mutations allow for some production of an internally deleted and somewhat functional protein.

- Potential therapies aim to turn Duchenne into **Becker muscular dystrophy (BMD)**, a milder, allelic disease whereby the reading frame is maintained and some dystrophin is produced.

- We have developed **MyoDys$^{45\text{-}55}$**, a CRISPR/Cas9 approach to delete *DMD* **exons 45-55** [1-2], that restores the reading frame for 50% of patients and creates a deletion associated with one of the more mild BMD phenotypes [3-4].

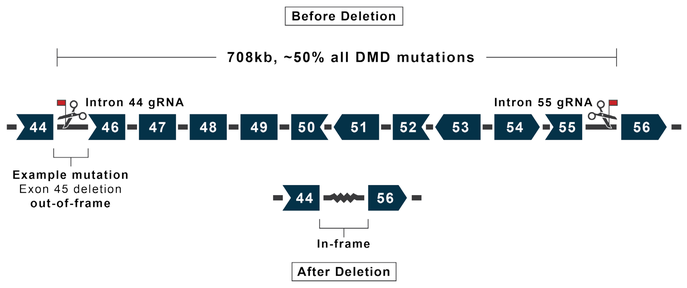

Figure 2: CRISPR/Cas9 to delete exons 45-55 for DMD. A schematic of a region of the *DMD* gene where exons are shown with blue boxes. gRNA (flags) targeting of Cas9 (scissors) to complementary sequences in introns 44 and 55 results in double stranded breaks that can be repaired through NHEJ, thereby deleting the intervening exons (~708kb) and restoring the reading frame for patients who have mutations in this region. The shape of the exon edges represent the reading frame [described in 2].

- **Adeno-associated virus (AAV)** is one method for delivery of CRISPR/Cas9 that can target muscle but has limitations due to the high dose required and the innate and adaptive immune response that occurs to AAV.

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OBJECTIVE: Assess immune suppression regimens for their ability to allow AAV redosing *in vivo*

- Immunosuppression (IS) can dampen the immune response to AAV and has been shown to allow redosing in preclinical models and one clinical trial with local AAV administration [5-6].
- Here we tested different combinations of IS drugs for their ability to allow redosing of systemic injections of AAV9-GFP followed by AAV9-mCherry.
- We removed individual IS drugs to assess the effect on redosing to determine which are essential. We also performed single cell RNA sequencing on PBMCs to understand the immune response.

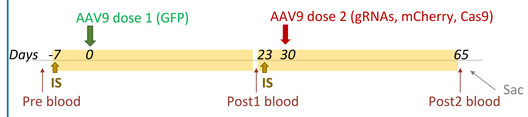

Figure 3: Timeline of experiment. Mice were treated with the IS regimen starting 1wk before the first AAV injection of GFP followed by an injection of AAV-mCherry a month later. Blood and PBMCs were taken at pre, post 1 and post 2 timepoints.

PRELIMINARY DATA

AAV9-MyoDys$^{45\text{-}55}$ restores dystrophin in a humanized DMD mouse model

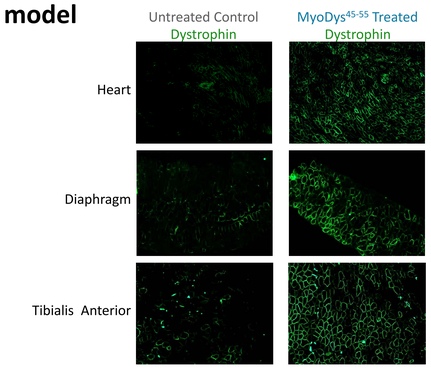

Figure 4: AAV9-MyoDys$^{45\text{-}55}$ treatment restores dystrophin in vivo. We have demonstrated AAV-mediated delivery of our CRISPR therapy can restore dystrophin in muscle *in vivo.* A single systemic injection of dual vector AAV9-MyoDys$^{45\text{-}55}$ (consisting of one vector containing CMV-Cas9 and one containing the two guide RNAs) was injected in P3 hDMD del45 mdx pups [1]. Dystrophin (green) immunostaining was assessed on heart, diaphragm and tibialis anterior muscle sections 7wks post-treatment.

Mdx mice function as model of AAV rejection

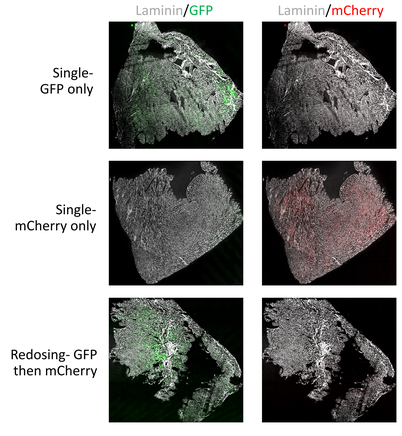

Figure 5: Mdx mice without immune suppression only express the first AAV injection. Single or dual injections of AAV9-GFP and AAV9-mCherry were injected systemically in adult mdx mice without immune suppression. Mice were given AAV-GFP alone (top row) , AAV-mCherry alone (second row) or redosed AAV where first AAV-GFP was given then AAV-mCherry was given 1 month later (bottom row). GFP (green) and mCherry (red) expression was assessed in hearts where muscle cells were outlined by laminin (gray). When redosing AAV without immune suppression only the first injection (GFP) is expressed thus demonstrating rejection/neutralization of the second injection.

RESULTS

Certain immune suppression regimens allow for AAV redosing

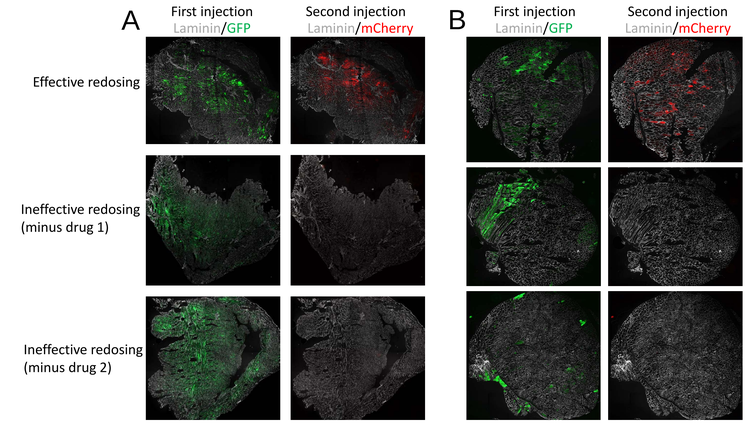

Figure 6: Redosing of AAV with immune suppression in vivo. Cohorts of adult mdx mice were given different immunosuppression (IS) regimens and systemic injections of AAV9-GFP and -mCherry as in Fig 3. Hearts (A) and triceps (B) were assessed for GFP (green) and mCherry (red) and muscle cells were marked by laminin (gray). An example IS regimen that allowed for redosing is shown (top row). Removal of individual IS drugs from that regimen resulted in ineffective redosing (second and third row) demonstrating those targets may be required in order to allow for effective redosing.

Single cell RNA-seq of PBMCs shows differential gene response

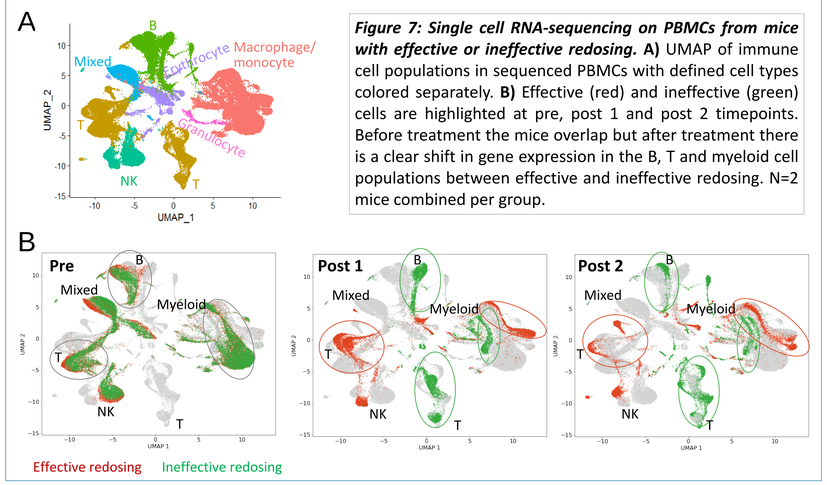

Figure 7: Single cell RNA-sequencing on PBMCs from mice with effective or ineffective redosing. **A)** UMAP of immune cell populations in sequenced PBMCs with defined cell types colored separately. **B)** Effective (red) and ineffective (green) cells are highlighted at pre, post 1 and post 2 timepoints. Before treatment the mice overlap but after treatment there is a clear shift in gene expression in the B, T and myeloid cell populations between effective and ineffective redosing. N=2 mice combined per group.

CONCLUSIONS AND ONGOING WORK

- Systematic assessment of the required IS drugs for redosing highlights the importance of targeting certain aspects of the immune system.
- There is a clear difference in the immune response as assessed by single cell RNA-seq with and without effective redosing.
- Although mice have not been considered to be a good model for the immune response, they are able to function as a basic model of AAV rejection/neutralization, however further work comparing to large animal models and humans is required.
- Ongoing work is testing novel IS targets for AAV redosing in mice.
- Ongoing studies are assessing dystrophin after redosed AAV-MyoDys$^{45\text{-}55}$.