COMMENT

CRISPR editing as a therapeutic strategy for Duchenne muscular dystrophy—anti-Cas9 immune response casts its shadow over safety and efficacy

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Duchenne muscular dystrophy is a progressive disorder that affects primarily boys, impacts mainly cardiac and skeletal muscle, and is caused by variants in the X-linked Dystrophin (*DMD*) gene [1]. DMD encodes a membrane associated protein that serves as a bridge between the extracellular matrix and the cytoskeletal network within the sarcoplasm, and its loss is associated with impaired membrane integrity and abnormalities in multiple signaling pathways [2]. The disease course is largely consistent across affected individuals, with onset around age 2 years, progressive proximal muscle dysfunction leading to loss of ambulation between ages 12–14, and continued muscle weakness and wasting associated with increasing respiratory and cardiac impairments. The implementation of glucocorticoid therapy, along with changes in respiratory and cardiac management, have led to improvements in quality of life and extension of life expectancy into the 30s [3]. However, DMD remains a severe, fatal disorder with a very high unmet need for disease modifying therapies.

DMD is a disorder well suited for genetic therapies [4, 5]. Pathogenic variants largely come in two flavors, nonsense mutations that promote nonsense mediated decay, and full exon (or multi exon) deletions that shift reading frame and result in frameshift and stop gain. The consequence at the molecular level is loss of Dystrophin protein. Previous therapeutic programs have concentrated on drugs that promote read-through of premature stop codons or ones that promote exon skipping to restore gene reading frame. These approaches have been only marginally successful, largely due to failure to restore meaningful levels of Dystrophin protein [6]. More recently, gene replacement therapy has emerged as an excited potential strategy [7]. Since DMD is too large for conventional AAV-based packaging, miniaturized versions of DMD are being tested. Currently there are 4 ongoing clinical trials evaluating different micro dystrophins, with results likely in the near future.

Due to the nature of the variants impacting the DMD gene, and owing to its large size, CRISPR mediated gene editing is an attractive alternative genetic strategy for treating DMD [8]. Various CRISPR based approaches have been considered, including creation of indels that abrogate splicing and promote exon skipping (promoted by non-homologous end joining), and precise editing to correct individual mutations, either using conventional Cas9 plus donor repair template or else using Prime or base editors (fusion enzymes of Cas9 plus deaminase) to precisely alter individual nucleotides. The most frequently pursued strategy to date has been to use CRISPR/Cas9 (delivered by AAV) to promote exon skipping, and data in pre-clinical models has shown highly efficient exon skipping, restoration of reading frame, and generation of Dystrophin protein. Several groups have shown success, including studies in patient cell, mouse, and canine models of DMD [9–14]. As compared to antisense oligonucleotides, CRISPR based exon skipping offers the advantages of a single administration, more extensive target delivery, and potentially more robust Dystropin restoration (though no head-to-head evaluations have been done).

Several concerns have been raised related to the translation of CRISPR/Cas9 based therapeutic approaches to patients. There are questions related to unintended editing events [15–17]. There are issues with cutting and repair efficacy, particularly in vivo, where efficiencies appear much lower than what is observed in cell culture. The systems used for delivering CRISPR reagents also have important safety considerations. AAV-based delivery appears to be the current standard for systemic administration, and dose dependent side effects of AAV are well recognized [18], including transient thrombocytopenia and transaminitis. While these events have proven largely manageable and not associated with longer term harm, infrequent but more serious adverse events have been reported with AAV gene therapy, including myocarditis and liver failure.

Perhaps the most concerning and challenging issue facing the field relates to the immunogenicity of the CRISPR machinery and particularly the editing nucleases. This is the subject of an excellent study by Duan et al. appearing in *Nature Communications* [19]. In this study (see Fig. 1), the Duan group tested both intramuscular and intravenous delivery of Cas9 and guide RNA into three different canine models of DMD. They found that AAV mediated expression of CRISPR reagents promoted editing of the DMD gene (e.g., small indels created at the site of Cas9 targeting by non-homologous end joining), but also resulted in both B- and T- cell based immune response to Cas9, the consequence of which was to reduce the extent and duration of Dystrophin restoration.

The authors began their study by enumerating the presence of existing humoral immunity to Cas9 in dogs, where they describe

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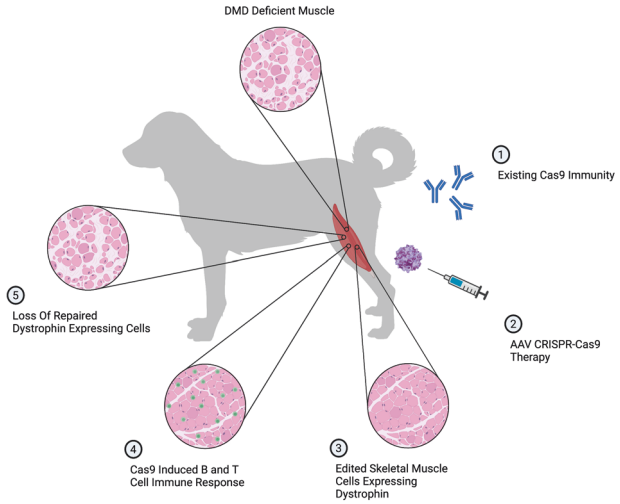

Fig. 1 Anti-Cas9 immunity and its impact on CRISPR editing in a DMD dog model. Schematic representing the major findings of Hakim et al. [19]. The authors studied canine models with mutations in the *DMD* gene that result in muscular dystrophy. (1) A high incidence of anti-Cas9 antibodies was observed in their dog colony. (2) They treated both young and old DMD dogs that had evidence of anti-Cas9 immunity with AAV encapsulated Cas9 plus guide RNAs, using both intramuscular and systemic infusion. (3) They observed robust gene editing with this strategy. (4) However, both humeral and cytotoxic T cell immune responses were observed. (5) This resulted in progressive loss in skeletal muscle of viral genomes and Cas9 expression, presumably through local immune destruction and clearance of infused myofibers, and (at least with intramuscular injection), reduction of Dystrophin re-expression. (Figure generated using BioRender and based on Gough and Gersbach [25]).

high levels of anti-Cas9 IgG in adult dogs, with much lower antibody levels in puppies. They then looked at local micro-injection of AAV-based expression of Cas9-guide RNA introduced into 1 month old dogs. They observed robust repair of the *DMD* gene, with restoration of Dystrophin protein, as has been previously observed in dog and mouse DMD models. However, they also detected local inflammation, with CD4 and CD8 cell infiltration into muscle, and signs of systemic immune response, with high levels of serum cytokine production. To study longer term consequences of the Cas9 immunity, they next injected AAV encapsulated Cas9 plus guide RNA into older dogs with existing Cas9 antibodies (44 months old), and found significant immune infiltration plus time related reduction of viral vector genomes, Cas9 transcript, and Dystrophin expression (peaking at 3 weeks post injection and nearly absent at 6 weeks). The likely basis for this is immune mediated destruction and elimination of Cas9 expressing myofibers.

The authors then performed a series of controls that demonstrated that the immune response was to Cas9, and not to other components of the delivery system. These controls included use of two different Cas9s, use of different AAVs, and delivery via AAV of non-Cas9 cargo (micro dystrophin and SERCA2A). They also used a muscle specific promoter to restrict Cas9 expression to skeletal muscle. The summary of these experiments is that only Cas9 induced an immune response. Of note, the authors trialed high dose prednisolone as an immune suppression strategy, and this did not prevent the cytotoxic T cell response nor the blunting of Dystrophin re-expression.

Lastly, systemic infusion was examined. In 1 month old DMD dogs, systemic administration of CRISPR reagents promoted extensive editing of the *DMD* gene with restoration of expression, and also elicited a robust B and T cell immune response. This correlated with reduction over time of vector genomes within the muscle and of Cas9 expression, though there was not a comparable reduction of Dystrophin re-expression. An immune response was also observed in wild type dogs infused with AAV-Cas9; while progressive reduction was seen in viral genomes, Cas9 transcripts, and levels of a co-infused marker gene (alkaline phosphatase), there was less cellular infiltration into the muscle and reduced levels of serum inflammatory markers.

In total, this study demonstrates that dogs with existing immunity mount an extensive B and T cell response to Cas9 that results in inflammatory infiltration into the muscle with reduction in Cas9 levels and, at least with intramuscular treatment, blunting of Dystrophin re-expression. Of note, similar data have been reported in mice that have pre-existing anti-Cas9 antibodies [20]. There are some important limitations of the study. Only a limited number of dogs were examined, though the responses were very consistent across the different treatment groups. Only two time points were examined, so understanding whether there are better or worse windows for exposure to Cas9 based therapy was difficult to extrapolate. All adult animals had existing immunity, so the potential impact on animals without anti-Cas9 antibodies is not clear. Lastly, the impact on Dystrophin re-expression with systemic treatment was unclear, as the authors did not observe the same dramatic reduction in Dystrophin as seen with intramuscular injection. This may be a reflection of the small sample size and/or the timing of assessment, and the reduction of viral genomes and Cas9 levels would imply immune mediated attack on Cas9 expressing muscle fibers and the eventual loss of Dystrophin re-expression. However, it is also possible that the consequence(s) of immune response with systemic administration some how differ from that of intramuscular treatment. Resolving this difference will be critical, given the potential impact on efficacy in patients.

Caveats aside, these data have important implications when considering translation of CRISPR editing to patients. High levels of existing adaptive immunity to different Cas9 species have been described in humans [21, 22], including 78% with anti-SaCas9 (S. aureus) antibodies and 78% with anti-Cas9 T cells reported in one comprehensive study [22]. These have largely been adult surveys, so it is not yet well established how prevalent Cas9 immunity is in the pediatric population. It is clear, though, that presence of existing immunity poses significant problems for safe and effective CRISPR based therapy. It may prevent any meaningful treatment response, either by immediate neutralization from existing antibodies, or subsequent immune "attack" on cells that have taken up and expressed the CRISPR machinery. Not only will the latter reduce/eliminate the Cas9, it will likely eliminate cells that have been successfully repaired. Also, the immune response carries with it potential harm to the individual, either from local inflammatory disease (myositis in the case of DMD) or from the systemic immune response.

There is therefore a critical need to develop strategies to overcome the problem of Cas9 immunity. Ex vivo approaches (i.e., introducing Cas9 to cells and then re-introducing them to patients), such as was recently done for sickle cell disease and beta thalassemia [23], does not appear to induce an immune response. However, this approach will not work at present for disorders of skeletal muscle, as cell transplantation has not been successful. Immunosuppression protocols may help, though the data from the current paper cast some doubt on the effectiveness of this approach (at least in terms of prednisolone alone, though combinatorial immune suppression may well be more effective). Newer generation synthetic nucleases may provide a solution, as they may not be targeted by pre-existing immunity. Lastly, an attractive approach is to consider creating immune tolerance to Cas9, such as through the generation and/or transfer of Cas9-specific Treg cells [24]. In all, whether one or a combination of strategies will be needed, overcoming the potential immune response to Cas9 is a key hurdle that will need to be cleared in order for CRISPR based therapeutics to be safe and effective.

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JJD conceived, wrote, edited, and finalized all aspects of this manuscript. Zachary Coulson helped generated the figure (see acknowledgements).

COMPETING INTERESTS

The author declares no competing interests.

ADDITIONAL INFORMATION

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