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Immune Responses to Muscle-Directed Adeno-Associated Viral Gene Transfer in Clinical Studies

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Muscle-directed gene therapy with adeno-associated viral (AAV) vectors is undergoing clinical development for treating neuromuscular disorders and for systemic delivery of therapeutic proteins. Although these approaches show considerable therapeutic benefits, they are also prone to induce potent immune responses against vector or transgene products owing to the immunogenic nature of the intramuscular delivery route, or the high doses required for systemic delivery to muscle. Major immunological concerns include antibody formation against viral capsid, complement activation, and cytotoxic T cell responses against capsid or transgene products. They can negate therapy and even lead to life-threatening immunotoxicities. Herein we review clinical observations and provide an outlook for how the field addresses these problems through a combination of vector engineering and immune modulation.

Keywords: adeno-associated virus, skeletal muscle, immune response, CD8 T cell, antibody

INTRODUCTION

GENE THERAPY DRUGS BASED ON adeno-associated viral (AAV) vectors have received regulatory approval for treatments of several genetic diseases by *in vivo* gene transfer, including Leber's congenital amaurosis, spinal muscular atrophy (SMA), and hemophilia A and B.[1,2] However, immune responses against vector or therapeutic transgene products continue to complicate AAV gene therapies, despite this vector's low innate immunogenicity compared with many other delivery systems.[3,4] Natural infection with AAV creates pre-existing humoral and T cell immunity in the human population. Multiple factors such as vector dose and design, target organ, and route of administration determine the risk of B and T cell activation after vector administration.

Skeletal muscle is an attractive target tissue for *in vivo* gene transfer owing to ease of access, long life span of muscle fibers, and the ability to secrete proteins into circulation. In fact, the first target in clinical gene therapy for hemophilia B was skeletal muscle, and the first approved AAV gene therapy product, Glybera, to treat lipoprotein lipase (LPL) deficiency, was also administered intramuscularly.[5] Moreover, skeletal muscles (especially diaphragm), cardiac, and other muscles are critical targets for correcting muscular dystrophies, certain lysosomal storage diseases, and other neuromuscular disorders by gene therapy including gene editing.

IMPACT OF ANTIBODIES AGAINST CAPSID ON EFFICACY OF INTRAMUSCULAR AAV ADMINISTRATION

One of the major hurdles to AAV-mediated gene augmentation is the presence of pre-existing neutralizing antibodies (NAbs) against the viral capsid.[6] About 80% of the human population develops such antibodies against

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various serotypes (starting during childhood), with prevalence varying substantially depending on capsid and geography.[7–9] One would expect high titers of pre-existing NAbs to preclude patients from receiving gene therapy with the respective serotype/capsid sequence. However, the impact of NAbs on muscle gene transfer is less clear as conflicting results have been reported from different clinical studies.

For example, a clinical trial in hemophilia B patients showed that intramuscular administration of AAV2 encoding human *factor 9* gene led to successful transduction and transgene expression despite the presence of pre-existing NAbs (titers ranging from 1:10 to 1:1,000).[10] Similarly, in clinical trials with intramuscular injection of alipogene tiparvovec (Glybera), more than half of patients (15/26) had pre-existing antibodies to AAV1, yet most of these patients achieved LPL expression.[11]

However, in one of the earliest phase I study in α1-antitrypsin (AAT)-deficient patients, only 1 patient out of 12 had transient and subtherapeutic levels of AAT in serum.[12] This was attributed to the presence of pre-existing antibodies against AAV2 capsid in almost all of the patients. In a follow-up clinical study, nine AAT-deficient patients were treated with intramuscular injections of AAV1-AAT over a range of doses.[13] Of note, four patients had previously been injected with the AAV2-AAT vector. Three of these patients received the same vector dose of AAV1-AAT as AAV2-AAT, whereas a fourth patient was given a higher dose.

In the intermediate group, two patients (previously injected with AAV2-AAT) achieved subtherapeutic levels of AAT in plasma that lasted up to 90 days in one patient and up to a year in the other. In clinical trials for different forms of limb-girdle muscular dystrophy (LGMD) using AAV1 vector, patients with pre-existing AAV1 NAbs nonetheless showed transgene expression in muscles fibers.[14–16] Subsequent treatment-emergent/increase of NAb titers did not affect transgene expression.[12,13,17]

Although not entirely conclusive, a trend emerges that pre-existing humoral immunity against AAV capsid is less of an obstacle for gene transfer by direct intramuscular injection. In contrast, NAbs that form after gene transfer are likely to prevent readministration. Therefore, immune suppression protocols are being explored to prevent NAb formation upon initial gene transfer. For instance, B cell depletion using anti-CD20 combined with mTOR inhibition with sirolimus (rapamycin) to target B and T cells is being tested in patients with Pompe disease.[18,19] Switching capsid sequence for readministration may be helpful within limitations.

For example, in a preclinical study with nonhuman primates, Greig et al. showed that readministration using heterologous serotype is possible by intramuscular administration when NAb titers are below a certain level at the time of second vector administration.[20] In contrast, pre-existing humoral immunity is more likely to negatively impact AAV transduction after intravenous administration of AAV vector, thereby complicating systemic vector delivery to muscle.[18]

CAPSID-SPECIFIC CELLULAR RESPONSES UPON INTRAMUSCULAR AAV ADMINISTRATION

The first indication of a cytotoxic T cell response to AAV gene therapy was observed in a clinical study of AAV2-mediated liver gene transfer of human coagulation factor IX in hemophilia B patients.[21] In this study, a patient in the high-dose cohort lost factor IX expression after initially achieving therapeutic levels. This was accompanied by a transient and self-resolving increase in transaminases. Further studies implemented a capsid-specific $CD8^+$ T cell response, which was not observed in preclinical studies.[22] Immune suppressive regimens (prophylactic or on demand), primarily based on steroid drugs, are now being employed and, to some extent, are successful in controlling/preventing the elicitation of cellular responses.[23] $CD8^+$ T cell responses against capsid were also observed in muscle gene transfer.[12–17]

Although inflammatory responses and T cell infiltrates were observed in muscle biopsies of patients treated with AAV1-AAT, these did not severely affect transgene expression long term and eventually diminished concomitant with the recruitment of $FoxP3^+$ regulatory T cells (Treg).[13,17,24] Interestingly, Mueller et al. showed the persistence of AAV capsids at the site of injection for up to 12 months after vector administration.[24] The presence of AAV capsids long after vector injection may mimic a state of chronic viral infection. Cellular infiltrates were observed to express PD-1 and PD-L1, perhaps representing an exhausted phenotype.

However, the potential for reactivation of such T cells is unclear. Apoptosis of infiltrating mononuclear cells at the site of vector administration was also observed in clinical trials for LGMD.[14,15] It should be pointed out, however, that patients were given immunosuppressive drugs before vector administration in these studies.

In LPL gene transfer, one of the patients had elevated levels of creatine kinase in plasma 1 month after vector administration, which corresponded with a decline in transgene expression.[25] High number of AAV capsid-specific T cells as estimated by enzyme-linked immunosorbent spot (ELISpot) assay on the peripheral blood mononuclear cells of this patient suggested the rejection of AAV transduced muscle fibers by T cells.[25] Furthermore, this study demonstrated a clear difference in the kinetics of activation of AAV capsid-specific $CD8^+$ T cells at low- and high-dose cohorts wherein at low vector dose, activation of $CD8^+$ T cells occurred at around 3 months and at high dose activation occurred around 1 month postvector administration.

TRANSGENE-SPECIFIC ADAPTIVE IMMUNE RESPONSES IN INTRAMUSCULAR AAV ADMINISTRATION

Although not as frequent, transgene-specific cellular immune responses are also a potential concern to the success of AAV gene therapy. This is particularly the case in treating Duchenne muscular dystrophy (DMD), where progressive degeneration and wasting of muscle fibers establish a proinflammatory environment in the muscle architecture. In a phase I trial with AAV2.5 vector encoding a truncated but functional version of the dystrophin protein, Mendell et al. found that two of the three patients in the high dose (1×10^{11} vector genomes [vg]/kg) cohort developed revertant dystrophin-specific T cell responses, whereas the third patient had a T cell response against minidystrophin.[26]

One patient in the low-dose (2×10^{10} vg/kg) cohort also had pre-existing cellular responses against revertant dystrophin that seemed to accelerate the development of T cell response after gene transfer in this patient. Interestingly, despite the presence of vg in all these patients, muscle biopsies from only two patients (one in each cohort) had minidystrophin expression. Of these two patients, one (in the high-dose cohort) developed a delayed (day 60 postinjection) T cell response.

T cell responses against dystrophin before gene transfer may reflect the sporadic expression of dystrophin epitopes in revertant fibers. Surveys of larger cohorts of DMD patients indeed found such pre-existing T cell responses. These studies also indicated that the probability of developing dystrophin-specific T cell response increases with the age of DMD patients and early treatment with glucocorticoids could have an immune modulatory effect.[27,28]

However, in a clinical trial of Becker muscular dystrophy (BMD), patients in both dose cohorts (3×10^{11} vg/kg per leg, *i.e.*, 6×10^{11} vg/kg; and 6×10^{11} vg/kg per leg, *i.e.*, 1.2×10^{12} vg/kg) developed transgene (follistatin)-specific T cell responses.[29] Immunosuppression was employed with only limited success, indicating that superior immune modulatory regimens should be developed.

Perhaps not surprisingly, the underlying mutation of the defective gene is a major determinant of antigen-specific immune responses against the transgene product because it will govern whether neoepitopes are being presented after gene transfer. Hence, gene deletions, inversions, frameshift mutations, and early stop codons (nonsense mutations) are more likely to predispose to immune responses than missense mutations. Unexpectedly, however, rare cases of $CD8^+$ T cell responses against AAT in patients with AAT deficiency resulting from a missense mutation revealed the potential for polymorphic sequence differences between endogenous and transgene to be another source of neoepitopes.[30]

The possibility of antibody formation that could clear a secreted transgene product and thereby prevent therapy was illustrated in a clinical trial that aimed to use AAV gene transfer to skeletal muscle for systemic delivery of an antibody against HIV.[31] Even when using humanized backbone sequences, such ''anti-idiotypic antibodies'' may form against the antigen-specific variable part of the immunoglobulin. To prevent antibody formation against factor IX in muscle-directed gene therapy for hemophilia B, only patients with F9 missense mutations were included.[10] This requirement was dropped in hepatic gene transfer, which is more likely to result in immune tolerance due to the immune regulatory pathways that are active in the liver.[3,21,32]

IMMUNE RESPONSES UPON SYSTEMIC DELIVERY OF AAV VECTORS TO MUSCLE

Treatment of DMD and other neuromuscular disorders is only effective if the vector is delivered to multiple muscle groups, which requires systemic administration of large vector doses through a blood vessel. For instance, multiple clinical trials attempt to treat DMD using this approach.[33] This route not only exposes the vector to preexisting NAbs but also creates a source of immunotoxicities. At vector doses $\geq10^{14}$ vg/kg, complement activation has been observed in multiple patients, which was often associated with a decline in platelet counts and thrombotic microangiopathy (TMA), causing kidney damage (presumably from activation of endothelial cells) and hemolysis.[34]

Hence, investigators started adapting monoclonal antibody therapy against complement components (*e.g.*, anti-C5, eculizumab) as a mitigation strategy to overcome these events, which appear largely antibody dependent. Thus, the classical pathway of complement activation likely plays a critical role, although direct binding of AAV capsid to components of the complement system, such as iC3b, may further increase the response.[34,35] Complement activation, TMA, and liver toxicity have also been observed in systemic AAV9 gene therapy for SMA.[36–39]

Primary and memory antibody responses may contribute to complement activation by AAV–antibody complexes, which is unlikely serotype-specific. More work is needed to define the potential role of capsid structure *in vivo*, given that AAV capsids may differ in their kinetics of clearance and biodistribution.[40]

Dystrophin-specific $CD8^+$ T cell activation is another major problem in systemic gene therapy for DMD. In systemic AAV microdystrophin trials, it was found that a region that is present in microdystrophin but absent in DMD patients (due to deletion mutations) might have induced T cell responses.[26,34] Therefore, patients with such mutations are currently excluded from clinical

trials. This decision was made in part because of several incidents of myocarditis in DMD patients after AAV gene transfer, suspecting a T cell response to be the cause of this cardiac pathology.[41,42]

A recent study in nonhuman primates receiving AAV9 vector (designed to treat the lysosomal storage disorder Pompe disease, a disease that also severely affects muscle function) demonstrated the potential for $CD8^+$ T cell responses against a transgene product expressed in the heart to result in myocarditis.[43]

These high-dose systemic deliveries also pose a risk for severe liver toxicities as the liver functions as a sink for vector. Accumulation of high vector loads in the liver may trigger the targeting of hepatocytes by capsid-specific $CD8^+$ T cells or be associated with other toxicities through yet undefined mechanisms. Importantly, both liver toxicities and TMA (and other pathologies resulting from complement activation) can be fatal for the patient.[36,37,39,44,45] More detailed information on the incidence and outcome of these toxicities is not yet available in peer-reviewed publications but is expected to become available in the future.

CONCLUSIONS AND OUTLOOK

Muscle-directed gene transfer is an important path for treating various neuromuscular disorders and is also attractive for systemic protein delivery. However, a tendency to induce B and T cell responses against capsid and transgene products and immunotoxicities associated with high-dose systemic delivery to muscle pose serious hurdles. Novel capsids that are effective at lower doses and are detargeted from the liver will hopefully help avoid the latter complications.[46,47] Although monoclonal antibody treatments or other drugs targeting complement components are being adopted as an adjunct therapy, the efficacy of such modulators is yet to be discerned.

More mechanistic preclinical studies are needed to guide the clinical development of vectors with reduced risk of $CD8^+$ T cell activation and to generate more effective transient immune suppression protocols. For example, we know that innate immune signals are requisite for T cell activation and may be derived from the activation of pattern recognition receptors. Sensing of AAV genomes by the endosomal DNA receptor TLR9 promotes $CD8^+$ T cell responses through induction of interferon type I (Fig. 1),[48–51]

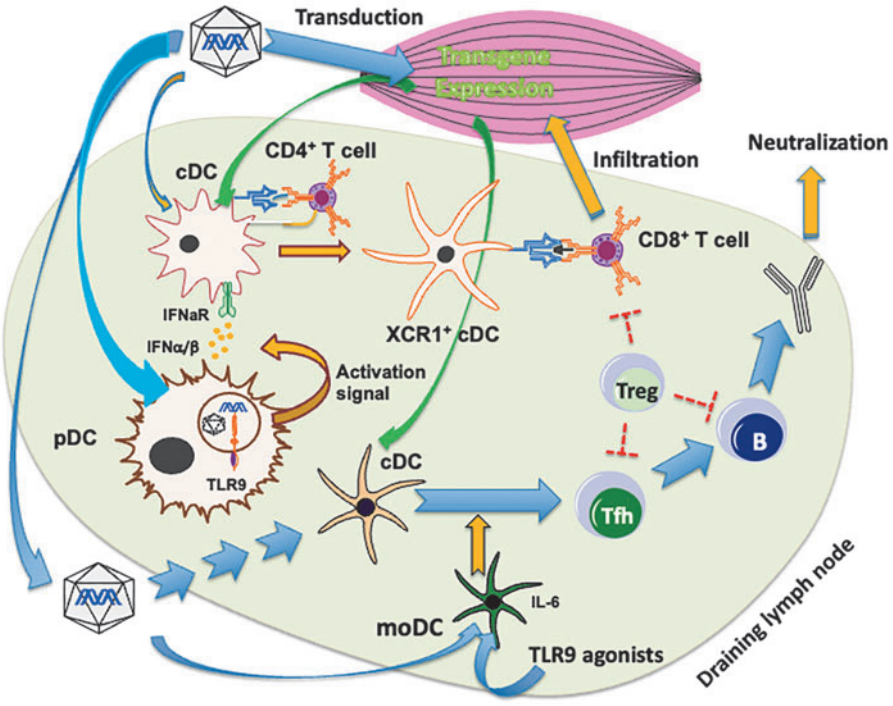

Figure 1. Known immune response mechanisms in AAV muscle gene transfer. The immune response starts locally in the draining lymph nodes of transduced muscle. TLR9 signaling in pDCs upon sensing vector genomes induces IFNα/β expression, which conditions cross-presenting cDCs. Combined with costimulatory signals from $CD4^+$ T helper cells, this leads to the priming of $CD8^+$ T cells against AAV capsid or transgene products. These may infiltrate transduced muscle and target muscle fibers that display peptides derived from capsid or transgene product through MHC I. Transport of the capsid or transgene product to dendritic cells in the T–B cell border may lead to activation of Tfh cells, which promote B cell activation and germinal center formation, leading to antibody formation, memory B cells, and plasma cells. moDCs activated by AAV or exogenous DNA enhance Tfh activation, thereby increasing antibody production. Other innate and cytokine signaling pathways driving B and T cell responses likely exist. Treg can limit B and T cell responses. Preventing TLR9 signaling (*e.g.*, through CpG depletion of vector genomes), eliminating transgene expression in dendritic cells (*e.g.*, by incorporation of miRNA target sequences into transcripts of the transgene), and blockade of cytokine signaling or costimulation represent some of the current and emerging approaches to prevent immune responses. AAV, adeno-associated viral; cDCs, conventional dendritic cells; miRNA, microRNA; moDCs, monocyte-derived/inflammatory dendritic cells; pDCs, plasmacytoid dendritic cells; Tfh, T follicular helper; Treg, regulatory T cells.

which already prompted vector developers to eliminate CpG motifs (which in their unmethylated form, such as in viral DNA, are potent agonists for TLR9) from transgene cassettes.[3,4,52–56]

Activation of monocyte-derived dendritic cells by AAV or exogenous DNA enhances antibody formation by increasing activation of T follicular helper cells, for example, by induction of IL-6 (Fig. 1).[57,58] Further basic research should aim to identify other targetable innate and cytokine signaling pathways that may be derived from the vector (pathogen-associated molecular patterns) or tissue damage (damage-associated molecular patterns) and promote B and/or T cell responses. Administration of steroid drugs is a conventional treatment for DMD and is widely used in clinical AAV gene therapy to prevent inflammatory and, more specifically, $CD8^+$ T cell responses. Regimens better tailored to AAV gene transfer, based on mechanistic considerations and large animal studies, can be developed.

Although AAV vectors are generally inefficient in transducing professional antigen-presenting cells (APCs such as dendritic cells), recent studies suggest that transgene expression in APCs occurs at sufficient levels to contribute to $CD8^+$ T cell activation[59–62] and that the use of muscle cell-specific promoters is insufficient to prevent this, especially at high vector doses.[63–66] Inclusion of microRNA target sites that result in the degradation of the transgene messenger RNA in APCs has been shown to be effective in murine models.[60] Transient *in vivo* inactivation of immunoglobulins or blockade of neonatal Fc receptors represent potential avenues to overcome preexisting NAbs.[60,67–69]

Going forward, the field continues to face challenges posed by the immune system. These are likely to extend to AAV delivery of bacterial nucleases in gene editing approaches.[70,71] Nonetheless, several highly promising avenues are being developed to manage the immunological hurdle. These include the development of novel vectors with improved efficacy and reduced accumulation in the liver combined with the incorporation of features that reduce innate immune signaling and antigen presentation into vector design, and the development of more tailored immune suppression regimens that are informed by mechanistic studies. Immune optimization of muscle gene therapy will increase safety, and extend gene therapy to more patients, even if they have unfavorable mutations.

AUTHOR DISCLOSURE

R.W.H. is serving on the scientific advisory board of the Regeneron Pharmaceuticals–Intellia Therapeutics collaboration on gene editing for hemophilia B, has served on a Biomarin roundtable on clinical gene therapy for hemophilia A, and has received funding from Spark Therapeutics for preclinical gene therapy studies. D.D. is a member of the scientific advisory board for Solid Biosciences and an equity holder of Solid Biosciences. D.D. is a member of the scientific advisory board for Sardocor Corp. D.D. is an inventor of several issued and filed patents on microdystrophin gene therapy and recombinant AAV vectors. The other author has no competing financial interests.

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