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The Skeletal Muscle Environment and Its Role in Immunity and Tolerance to AAV Vector-Mediated Gene Transfer

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Abstract

Since the early days of gene therapy, muscle has been one the most studied tissue targets for the correction of enzyme deficiencies and myopathies. Several preclinical and clinical studies have been conducted using adeno-associated virus (AAV) vectors. Exciting progress has been made in the gene delivery technologies, from the identification of novel AAV serotypes to the development of novel vector delivery techniques. In parallel, significant knowledge has been generated on the host immune system and its interaction with both the vector and the transgene at the muscle level. In particular, the role of underlying muscle inflammation, characteristic of several diseases affecting the muscle, has been defined in terms of its potential detrimental impact on gene transfer with AAV vectors. At the same time, feedback immunomodulatory mechanisms peculiar of skeletal muscle involving resident regulatory T cells have been identified, which seem to play an important role in maintaining, at least to some extent, muscle homeostasis during inflammation and regenerative processes. Devising strategies to tip this balance towards unresponsiveness may represent an avenue to improve the safety and efficacy of muscle gene transfer with AAV vectors.

Keywords

AAV vectors; muscle; clinical trials; immune responses; tolerance

INTRODUCTION

Recombinant adeno-associated virus (AAV) vectors (1) have gained broad interest as tools to achieve therapeutic gene transfer *in vivo* in a variety of models of disorders affecting muscle, brain, eye, and liver due to their excellent safety profile and their ability to transduce a wide variety of post-mitotic tissues, providing efficient and stable transgene expression (2). Recently, the first gene therapy drug based on an AAV vector injected

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CONFLICT OF INTEREST
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intramuscularly, Glybera, has been approved by the European Medicine Agency for the treatment of lipoprotein lipase deficiency (3).

Since the early days of gene therapy, skeletal muscle was considered as a potential target for *in vivo* genetic engineering to create a site for the production of secreted proteins following AAV vector-mediated gene transfer (4-9). However, muscle tissue is also the hotbed of immune reactions, and intramuscular injection is commonly used for vaccination purposes. As a consequence, local immune reactions need to be cautiously addressed upon gene delivery to muscle, as they may represent an obstacle to the success of therapies aiming at restoring normal protein expression in enzyme deficiencies (6-9) and hereditary muscular disorders (6-8, 10-17). In addition, the vast heterogeneity the disease state of muscle in neuromuscular disorders provides an additional layer complexity to the understanding of immunity in muscle gene transfer, since tissue remodeling and/or disease-related inflammation may impact the context in which either the vector or the encoded transgene will be presented to the immune system (18).

Finally, recombinant AAV vectors are derived from their wild-type counterpart, to which humans are exposed early in life (19-21). This results in development of both humoral (22, 23) and cellular (24) immunity to the vector capsid, which may prevent or reduce therapeutic efficacy following gene transfer.

In this review, we will focus on AAV-based gene transfer to skeletal muscle and highlight the limitations that could be encountered due to the immune response against the vector and/or the transgene.

IMMUNE RESPONSES DIRECTED AGAINST THE AAV CAPSID

Wild-type AAV is a replication-defective parvovirus initially isolated from preparations of viruses infecting humans through the airways (25). While no known pathology is associated with AAV infection, it is known that this small non-enveloped single-stranded DNA virus triggers both innate (26) and adaptive immunity (27, 28), resulting in long-term humoral and cellular immune responses against the structural proteins of the capsid. When it comes to gene transfer with AAV vectors, these immune responses can abolish transgene expression, either by neutralizing the vector before it reaches the desired target tissue (29) or by clearing the transduced cells (29-32).

Anti-AAV Neutralizing Antibodies

Following the exposure to the wild-type virus, a significant proportion of humans develop humoral immunity against the capsid early in life, starting around 2 years of age (19-21). Additionally, shortly after birth, maternal anti-AAV antibodies can be found in newborns (19), resulting in a narrow time window, if any, in which the majority of humans is naive to anti-AAV antibodies.

Because of the high degree of conservation in the amino acid sequence across AAVs (33), anti-AAV antibodies show cross-reactivity with a wide range of serotypes (22). In healthy donors, anti-AAV1 and -AAV2 antibodies appear to be the most prevalent (more than 60%

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of the population is seropositive to AAV2) and display the highest neutralizing titers (19, 21-23, 34). Conversely, about one third of healthy humans are seropositive for AAV8 and AAV9, and generally titers are low (22).

Anti-AAV antibodies can have a profound impact on the efficiency of tissue transduction with AAV vectors, as even low-titer antibodies can neutralize the virus following systemic delivery (29, 35-37), a route of vector administration used to target several tissues, including liver (29, 31, 32), muscle (13, 35), and central nervous system (38). Thus, pre-screening of subjects for pre-existing immunity to AAV prior to enrollment in clinical trials is a necessary step to guarantee therapeutic efficacy following AAV vector systemic administration. Conversely, neutralizing antibodies to AAV are not an issue when the vector is delivered to certain immunoprivileged body sites (e.g. to the subretinal space (39-41)) or intraparenchymally (Table 1). Importantly, while recombinant AAV1 vectors have been delivered via local injection into the skeletal muscle (Table 1), several gene therapy approaches in which AAV vectors are delivered intravascularly or systemically have been recently proposed to target skeletal muscle (13, 35, 42, 43), or myocardium (44), which makes the issue of pre-existing humoral immunity to AAV particularly relevant (Fig. 1). Notably, neutralizing antibodies to AAV constitute also a major obstacle to vector readministration, as they are elicited at high titers following gene transfer and these titers persist for several years. This is a particularly important issue when it comes to systemic disorders, like muscle disorders, in which whole body transduction is necessary and may require repeated vector administrations. Furthermore, the fact that AAV vectors do not integrate efficiently into the host genome and do not transduce satellite cells (the muscle stem cells) (45), together with the fact that transgene expression may not persist long-term in a diseased muscle (46), make safe and effective AAV vector readministration an important goal for the future.

To date, the only strategy adopted in clinical trials to prevent vector neutralization by anti-AAV antibodies has been to exclude seropositive subjects from enrollment. This approach is far from being optimal, as it prevents a large proportion of subjects from being enrolled in potentially beneficial clinical trials; furthermore, exclusion of seropositive subjects requires the use of reliable and sensitive antibody assays (Table 2).

To allow efficient vector delivery and transgene expression in seropositive patients, several strategies have been tested to lower preexisting humoral responses to AAV (28). These include pharmacological, physical, and capsid engineering methods, each carrying advantages and potential drawbacks. While examples of prevention of anti-AAV antibody following vector administration using immunosuppression are reported in the literature, for example by using a non-depleting anti-CD4 antibody (47, 48), achieving this goal in large animal models of gene transfer has been challenging even when intensive regimens were used (49). Similarly, eradication of anti-AAV antibodies via immunosuppression is likely to be difficult (34) and to require very aggressive drug regimens in order to target both B and T cells and in particular plasma cells; this raises concerns over possible toxicities related to immunosuppression, such as the increased risk of developing malignancies and infections, or over possible interactions of immunosuppressive drugs with gene transfer (50, 51). Plasmapheresis has recently gained attention as a clinically approved, safe method that can

be used to lower pre-existing anti-AAV antibodies. In humans, Monteilhet and colleagues showed that up to five cycles of plasmapheresis resulted in a dramatic drop in anti-AAV neutralizing antibodies (52), a result that was also confirmed in other studies (53, 54). While results obtained are promising, the technology likely requires some improvement, as it is non-specific, resulting in the depletion of all immunoglobulins, thus requiring supplementation with intravenous immunoglobulin (IVIg), and it is not very efficient, as it requires multiple cycles of immunoabsorption over time to remove circulating and extravascular IgG (52). Furthermore, the efficacy of plasmapheresis is limited when high-titer anti-AAV antibodies are present, such as in the context of vector readministration. The use of capsid decoys to shield AAV vectors from antibody-mediated neutralization has also been recently proposed (55), however this approach needs to be weighted against the potential issue of T cell immunity directed against the AAV capsid (30).

T Cell Responses Directed against the AAV Capsid

In addition to neutralizing antibodies, natural infection with wild-type AAV also triggers cell-mediated immune responses against the capsid, which results in a reservoir of memory $CD8^+$ T cells that can be reactivated upon vector administration. This can cause the destruction of transduced cells presenting AAV capsid antigen in the context of MHC class I (56, 57), as it has been observed in subjects enrolled in AAV vector mediated liver gene transfer trials (29-32). While several studies looked at humoral immunity to AAV, only few reports on T cell reactivity to AAV in healthy humans are available in the literature. Veron and colleagues used a lentiviral vector expressing the capsid protein VP1 to restimulate peripheral blood mononuclear cells (PBMCs) and evaluate T cell reactivity to AAV1 in healthy donors. The survey, performed both by IFN-gamma ELISpot and polyfunctional flow cytometry assay, showed a prevalence of capsid T cell reactivity of about 30% in humans (24). In another study, T cell reactivity to AAV2 was detected at higher frequency, >70%, in splenocytes collected from human subjects restimulated with peptides derived from the AAV VP1 protein (30, 58). These T cells showed a memory phenotype, and appeared to recognize specifically MHC class I epitopes against which reactivity was also found in subjects dosed with AAV vectors (30). Importantly, several of the identified AAV capsid MHC class I epitopes are highly conserved and cross recognized by T cells (30), making the relatively simple maneuver of switching to “non-human” serotypes of AAV vectors (33) to avoid memory T cell responses to the capsid unlikely to succeed. While the fact that AAV vector administration elicits T cell-mediated immunity is not completely surprising, as AAV vectors are essentially perfect copies of wild-type AAV to which humans are exposed, several open questions remain on the role of these capsid-specific $CD8^+$ T cells in the outcome of gene transfer, as detection of T cell reactivity to the capsid in PBMCs has not always been associated with detrimental effects on gene transfer in liver (31, 32) and muscle trials (Table 1).

Reemergence of memory anti-capsid $CD8^+$ T cell responses (or *de novo* stimulation of a cytotoxic response to the AAV capsid) following AAV administration has been extensively characterized in humans undergoing AAV vector gene transfer to the liver, in which an increase in liver enzymes and loss of transgene expression was documented (29-32). In a first clinical trial in which an AAV2 vector was introduced into the liver of severe

hemophilia B subjects (29), two subjects developed transient elevation of liver enzymes and loss of oagulation factor IX transgene expression around week 4 post vector delivery due to the immune rejection of transduced hepatocytes mediated by capsid-specific $CD8^+$ T cells (30). A similar set of observations was made in the context of another hemophilia B clinical trial in which an AAV8 vector was infused systemically (31, 32). These studies showed that, like for AAV2, AAV8 vector administration in humans results in activation of capsid-specific T cells, and in some cases this is associated with the loss of transgene expression and an increase in liver enzymes. In the AAV8 hemophilia trial, this phenomenon has been documented in 4 out 6 subjects dosed with 2×10^{12} vector genomes per kg (32) and has been controlled with a short course of steroids administered at the time of liver enzyme elevation and loss of transgene expression. Notably, timely administration of immunosuppression at the time of liver enzyme elevation resulted in better rescue of transgene expression, underscoring the need for careful monitoring of adverse immune responses in gene transfer (32).

Preclinical studies in small and large animal models of gene transfer failed to predict the outcome of gene transfer observed in human trials. In mice, induction of anti-capsid cytotoxic T lymphocytes (CTLs) responses directed against AAV2 vectors did not result in clearance of AAV2-transduced cells neither in the liver nor in the muscle (59-61), with the exception of a recent study in which $CD8^+$ T cells primed against AAV capsid epitopes were adoptively transferred into immunodeficient animals transduced with AAV vectors (62). Cellular immunoreactivity to the capsid has nevertheless been detected in a study in dogs following intramuscular administration of AAV2 and AAV6 vectors (63). In this study, immunosuppression was required to allow for long-term expression of the transduced myocytes (63).

In muscle gene transfer clinical trials, the emergence of CTLs against the AAV capsid after intramuscular administration of AAV vectors (Table 1) appears to be, at least to some extent, dose-dependent and is accompanied by the detection of T cell infiltrates in the injected muscle; in some instances, T cell reactivity to the AAV capsid has been associated with the apparent lack of transgene expression (64, 65).

Despite the detection of T cell reactivity against AAV in PBMCs and the identification of infiltrates in the muscle of AAV-treated patients, the transgene was still expressed in subjects who received an AAV1 vector encoding for alpha-1 antitrypsin (66-68). Interestingly, $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) were also found within the infiltrating cells in vector-injected muscle, which may account for the control of capsid cytotoxic T lymphocyte (CTL) responses locally (68). In this study, the persistence of AAV antigen locally in muscle and the expression of PD-1/PDL-1 by T cells may explain the expansion and maintenance of Tregs in muscle. Interestingly, it has been shown that alpha-1-antitrypsin has tolerogenic properties as, for example, it promotes expansion of regulatory T cells *in vitro* [69].

Treg infiltrates were also found in a study of AAV1 muscle gene transfer for lipoprotein lipase deficiency (Table 1), however the administration of an intensive immunosuppressive regimen in this study may also have affected the outcome of gene transfer (70, 71).

Nevertheless, data emerging from preclinical (72) and clinical (64) studies of direct intramuscular injection of AAV vectors indicate that the muscle has unique features when it comes to immune response to AAV gene transfer, which may result in apoptosis of reactive T cells and, therefore, allow for long-term transgene expression.

One caveat in the interpretation of results from several of the AAV trials in which muscle was targeted is that the measurement of endpoints of safety (e.g. muscle enzymes) and efficacy (transgene expression) has not always been straightforward. The presence of underlying muscle inflammation (73), the fact that several transgenes are not secreted or their effect not easily measurable (Table 1), and the fact that in some cases immunosuppression was used together with gene transfer (70, 71), further complicates the interpretation of results.

No conclusive data, no conclusive data are available on the impact of contaminants in AAV vector preparations on the immune response detected following gene transfer. However, improvements in vector design (74) and production processes (for example aimed at removing empty capsids) (56) may represent important steps towards the enhancement of the safety of gene transfer in humans.

Future studies will have to better decipher the factors contributing to vector immunogenicity in muscle gene transfer, and devise strategies to reduce the therapeutic dose of vector (75) to improve the efficacy of the approach while reducing potential immune-mediated toxicities.

IMMUNE RESPONSES DIRECTED AGAINST THE TRANSGENE PRODUCT: WHAT WE HAVE LEARNED FROM INTRAMUSCULAR VECTOR ADMINISTRATION

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Direct injection of AAV vectors has been extensively tested as a strategy to engineer the muscle to produce lacking enzymes (67, 76-78), to provide expression of proteins vital for muscle physiology (10, 64, 79, 80), or to correct metabolic defects (81). This work initially focused on AAV serotype 2 (77, 82), however today most gene therapy approaches rely on AAV serotypes such as 1, 6, 8, 9 (33, 83-85), and some derivative of these serotypes (10, 86-88), which target the muscle with higher specificity and efficiency than AAV2. From an immunology perspective, pre-clinical and clinical work on intramuscular muscle gene transfer has been extremely important to understand the role of transgene immunity in gene transfer, and to understand the roles played by the context of gene transfer, such as the genetic background of the host and the muscle environment itself.

THE EXPERIENCE WITH INTRAMUSCULAR GENE TRANSFER TO RESTORE METABOLIC DEFECTS AND ENZYME DEFICIENCIES

In gene-replacement therapy, the therapeutic transgene product encoded by the AAV vector is typically a neo antigen to which the host may lack central tolerance. This, and other concurring factors (Table 3), may result in transgene-specific immune responses and activate effector mechanisms mediated by either neutralizing antibodies (89) or T cells (7).

Initial gene transfer studies in mice were very encouraging as they showed that AAV2 vectors were able to allow for efficient and long-term expression of an immunogenic transgene in normal skeletal muscle following intramuscular injection. This outcome of gene transfer was strikingly different from what was observed in animals injected with adenoviral (Ad) vectors (4, 5, 90), in which a strong humoral and cellular immune response to the transgene was observed. Differently, little to no responses were observed following AAV vector intramuscular injection (91), and the difference in immunogenicity of Ad vs. AAV vectors was attributed to differences in the ability to transduce antigen-presenting cells (APC), particularly dendritic cells (DC), *in vivo* (92). These results were confirmed in studies in which stable expression was reported for transgenes like erythropoietin (4), alpha-1-antitrypsin (90), and coagulation factor IX (F.IX) (9) following intramuscular AAV vector administration. Further evidence that AAV vectors are less immunogenic than Ad vectors (89, 90) also comes from the observation that, when used as vaccines, AAV vector elicit weak, poorly protective $CD8^+$ T cell responses (93). Furthermore, when highly immunogenic proteins such as influenza hemagglutinin HA are expressed in the muscle with AAV vectors, strong cell-mediated immune responses are triggered, which results in the elimination of transduced fibers within four weeks from vector administration (94). However, in this setting the kinetics of induction of antigen-specific $CD4^+$ T cells appears delayed when the transgene is delivered with AAV as compared to Ad vectors, suggesting differences between the two vectors in either the signals delivered to the immune cells or the way the antigen is presented. Since no efficient transduction of DC has been observed with AAV vectors, uptake of antigen by APCs appears to be the prevalent mechanism of antigen presentation in the context of AAV gene transfer (94). Indeed, $CD8^+$ T cell activation against the GFP transgene was documented in AAV vector intramuscular gene transfer via cross-presentation of the antigen by dendritic cells (95). Nevertheless, some degree of transduction of DC *in vitro* has been evidenced also in dogs and humans (96, 97), and *in vivo* in mice (98). Further indirect evidence that the transduction of APC *in vivo* is a key determinant of the transgene immune responses in AAV gene transfer comes from studies in which detargeting of transgene expression in APC with mir142-regulated (99) AAV vectors abolished $CD4^+$ and $CD8^+$ T cell responses against an immunogenic transgene in mice (100). This strategy was effective in reducing immunogenicity of different transgenes (100-102), however it failed to prevent immune responses to the transgene in inflamed muscle, where cross-presentation of antigen at the DC level is presumably more efficient (103). Finally, recent work from Carpentier and colleagues suggests that $CD8^+$ T cell responses directed against transgenes in AAV gene transfer to the muscle are determined by the presence of MHC class I epitopes together with strong MHC class II epitopes and also B cell epitopes (101). Conversely, it has been shown that poor APC transduction associated with other factors such as T cell exhaustion and low upregulation of MHC class I molecules on target cells can result in induction of tolerance (104), suggesting that the combination of multiple parameters drives the balance between tolerance, ignorance, and immunity in muscle gene transfer.

Although transgene-specific $CD8^+$ T cells (105) and muscle infiltrates have been detected in several AAV muscle trials, the impact of cell mediated immunity on AAV-transduced myofibers has been questioned (72). Indeed, it has been shown that T cells infiltrating the

muscle display an exhausted phenotype, lose their cytotoxic potential, and eventually undergo apoptosis (64, 72). While this phenomenon does not seem to be associated with the induction of active tolerance to the transgene, as it is has been described for liver gene transfer with AAV vectors (106), more complex and complementary mechanisms may concur to shaping transgene immunogenicity in the muscle.

Several studies analyzed immune responses directed against the transgene in AAV gene transfer for coagulation factor IX (F.IX) to muscle in preclinical models of hemophilia B. This work allowed identifying the genetic background of the host as one of the crucial determinants of transgene immunogenicity (107-110), with the magnitude of the immune responses directed against the F.IX transgene directly correlated to the degree of disruption of the endogenous F.IX gene; particularly with *null* mutations resulting in the highest immunogenicity (107). Data in hemophilia B dogs are in agreement with these findings, and show that in intramuscular gene transfer with AAV vectors higher vector doses injected locally result in enhanced transgene immunogenicity (111). In addition to genetic background and local vector dose, recent studies in mice showed that self-complementary AAV1 vectors encoding for F.IX injected intramuscularly can lead to a stronger $CD8^+$ T cell response to the transgene compared to single-stranded vectors (108), a result in agreement with previous findings in the context of AAV gene transfer to the liver (112). These results are in agreement with the reported higher levels of transduction in DC with self-complementary vectors as compared to single-strand vectors after intramuscular injection in mice (98) and in *in vitro* in human DC (96)

The availability of animal models to test transgene immunogenicity in different gene therapy scenarios has been crucial for the design of clinical gene transfer trials (77, 78), in which only subjects with residual protein levels were enrolled and received multiple intramuscular injections of AAV vectors to maintain the local vector dose below a critical threshold for immunogenicity (111).

Finally, preexisting immunity to the transgene may be found in patients suffering from lysosomal storage diseases affecting the muscle, such as Pompe disease, in which immunity triggered against the therapeutic protein is relatively common (113). In hemophilia, eradication of pre-existing immunity to coagulation factor VIII and F.IX has been achieved with liver gene transfer with AAV vectors (114, 115); in intramuscular gene transfer, high doses of AAV vectors expressing F.IX resulted in induction of anti-transgene antibodies in mice, which were then cleared over time (116). Whether clearance of pre-existing humoral immunity to a protein via muscle directed gene transfer is a result consistently achievable remains to be tested.

One alternative strategy to reduce or avoid the induction of immunity to the transgene product, or to overcome preexisting immunity that would prevent gene transfer, is to overexpress a specific endogenous protein whose function can correct the disease phenotype. For example, activated coagulation factor VII (F.VIIa) has been used to achieve correction of the bleeding phenotype in dog models of hemophilia by expressing the transgene in the liver via AAV8-mediated gene transfer (117). While the approach may be feasible in muscle, it should be noted that supraphysiological levels of expression of a

transgene in muscle might break tolerance to the transgene itself. For example, an autoimmune response to erythropoietin (Epo) has been reported following intramuscular injection of AAV-Epo vectors in macaques (118).

The Experience with Neuromuscular Disorders

Considering the broad heterogeneity of muscular dystrophies, it is worth mentioning that it appears that gene expression profiles of hindlimb muscle is markedly different between severely and mildly affected animal models (119). Similarities in the expression profile of dystrophin- and sarcoglycan-deficient mice, including inflammatory and remodeling processes, were also found (119). A chronic inflammatory response characterized by local cellular infiltrates, including macrophages, is a shared feature in several dystrophic mouse models, and chemokines such as CCL2 and CCL6 correlate with macrophages and T cell recruitment in inflamed muscle (120). Therefore, the dystrophic muscle already contains all the signals and the components of a productive immune response. In dystrophic patients and in murine models of muscular dystrophies, anti-inflammatory and/or immunosuppressant drugs have a beneficial effect, further highlighting the role of the immune system in the disease pathophysiology (121-123). Studies in mice support the involvement of B and/or T lymphocytes in the course of Duchenne muscular dystrophy (DMD) and limb girdle muscular dystrophy (LGMD) type D (124, 125). Additionally, in the absence of immunomodulatory treatment, activated T cells were found in the muscle of the *mdx* mice, a model of DMD, and depletion of either $CD4^+$ or $CD8^+$ T cells resulted in an attenuation of the disease (126). These results correlate with findings in humans, as muscle biopsies from DMD patients show the presence of clonal populations of T cells with conserved TCR sequences (127), and T cell reactivity directed against dystrophin was recently documented in a large proportion of DMD patients, with over 50% of the subjects showing a positive signal in PBMC restimulated with peptides derived for dystrophin and tested in an IFN-γ ELISpot assay (73). T cell reactivity in these subjects appeared to increase with age and was decreased in subjects treated with corticosteroids (73).

In dog models of muscular dystrophy, systemic delivery of an AAV9 vector constitutively expressing minidystrophin to neonate animals resulted in widespread muscle transduction (as predicted in prior studies conducted in neonate dogs with a reporter transgene (128)) and detection of transgene-positive myofibers. The procedure, however, was also associated with marked inflammation and consequent muscle atrophy and contractures (83). This prompted the use of immunosuppressive regimens (129, 130) to prevent or lower immune reactivity following intramuscular gene transfer (for a review, see (131)). Optimization of the microdystrophin sequence, together with the use of a muscle-restricted promoter and serotype 8 AAV vectors has been shown to reduce the immunogenicity of the approach (132), as shown also in a recent study of AAV8 vector mediated exon skipping in Golden Retriever Muscular Dystrophy dogs (13).

In clinical gene transfer trials, dystrophin immunity in DMD was documented in a clinical trial of intramuscular gene transfer with an AAV2.5 vector (a hybrid vector between AAV1 and AAV2 (10)) encoding for mini-dystrophin in DMD subjects (Table 1, (105)). In this study, apparent loss of transgene expression following gene transfer was associated with

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detection of T cell reactivity against $CD4^+$ and $CD8^+$ T cell epitopes in the mini-dystrophin transgene that were not present in the endogenous dystrophin gene due to the underlying disease causing mutation. Notably, baseline reactivity to dystrophin was also detected, indicating the presence of immune reactivity directed against revertant fibers. Revertant fibers are found DMD patients and are thought to arise form somatic mutations in the revertant nucleai, which restore frameshift mutations originating from small deletions in the dystrophin gene (133). Unlike in *mdx* mice (134), in humans, revertant fibers do not seem to increase over time, possibly reflecting immune mediated clearance of newly-formed fibers, and their detection is not associated with a clinically relevant improvement in the disease course (135). Together, findings on revertant fibers and the basic pathophysiology of DMD should be taken into careful considerations in the design of future gene transfer trials, in which inflamed muscle is being targeted and immunomodulation may be necessary to modulate preexisting transgene-directed immune responses.

As in the case of metabolic diseases (117), also for muscle diseases it might be possible to overexpress endogenous muscle proteins to achieve correction of the disease phenotype. This approach was recently tested in humans in a phase I/II clinical trial of AAV1 vector-mediated gene transfer for follistatin in Becker muscular dystrophy patients (136), which showed amelioration of the disease phenotype. Similarly, overexpression of utrophin, a dystrophin-related protein, has been proposed as an alternative for the treatment of DMD (for a review see (137)). In *mdx* transgenic mice over-expressing utrophin, a significant amelioration of the disease phenotype was observed (138); similarly AAV vectors have also been used to deliver to the muscle a functional truncated form of the utrophin (139) or upstream factors involved in the upregulation of the utrophin expression, such a PGC-1α and Jazz (140, 141). These strategies could allow for avoidance of immune responses to dystrophin, as it has been observed in the context of Adenovirus-mediated gene transfer (142). However, it should be kept in mind that the structural organization of dystrophin vs. utrophin differs significantly in muscle (143), arguing for the potential limitation in full correction of muscle function with this approach.

The Muscle Environment and Transgene Immunogenicity

Many different immune cells involved in innate immunity contribute to the pathogenesis of dystrophies by intervening either in the degenerative or regenerative process of the muscle characteristic of this family of diseases, these cells include neutrophils, eosinophils, macrophages, mast cells (for review, (144, 145)). Early depletion of myeloid cells in *mdx* mice have been reported to decrease necrotic fibers by 70-80% (146), probably by containing the effects of proinflammatory M1 macrophages, which can worsen the disease by promoting muscle damage. Conversely, anti-inflammatory M2 macrophages seem to play an essential role in muscle regeneration: M2a macrophages reduce the lytic activity of M1 macrophages, while M2c macrophages promote muscle regeneration (145). These populations of macrophages are highly versatile (147) and can be influenced by the cytokine milieu. The cross talk between T cells and macrophages in fact plays a role in muscle pathology of DMD (144) and may also play an important role in AAV vector-mediated gene transfer (Fig. 2). Both DC and macrophages are able to present transgene antigen to $CD4^+$ T helper cells in draining lymph nodes of dystrophic and healthy muscle; however in

dystrophic muscle antigen presentation is enhanced (103). As a result, an increase in transgene immunogenicity may be observed in dystrophic vs. normal muscle (Fig. 2). Indeed, increased immunogenicity of the reporter transgene β-galactosidase expressed with an AAV2 vector was observed in dystrophic mice compared with wild-type animals (148).

Concomitant to enhanced antigen presentation, a high concentration of macrophages reactive against myofibers (50,000 macrophages/mm^3) is found in muscle of *mdx* mice (146). These macrophages may influence the induction of T cell-mediated responses in gene transfer. Indeed, we found that macrophages were at least partially involved in the immune-mediated loss of transgene expression following AAV vector-mediated muscle gene transfer in a murine model of LGMD type D (103). $CD8^+$ T cells also contributed to the elimination of transduced muscle cells in dystrophic mice but not in normal mice (103).

Changes in the immune environment during muscle injury also include anti-inflammatory responses. For example, the anti-inflammatory M2c macrophage subpopulation that emerges during muscle regeneration after injury responds to IL-10 (149). Interestingly, a population of muscle-specific regulatory T cells (150) have been identified in dystrophic muscle of *mdx* mice and also detected in muscle infiltrates from DMD patients (151). These IL-10 secreting Tregs improve the dystrophic phenotype by decreasing inflammation associated with the disease, and their depletion results in worsening of the disease phenotype, while induction of Tregs with IL-2 has a beneficial effect (151). Thus, treatments soliciting the emergence of a stable Treg population in muscle at the time of gene transfer may reduce muscle inflammation and favor maintenance of transgene expression. Indeed, administration of exogenous transgene-specific Tregs concomitantly to AAV vector gene transfer was shown to lower anti-transgene immune reactivity and to allow stable transgene expression (152) in normal muscle. Moving forward it will be important to evaluate the efficacy of the approach in a dystrophic context, since inflammatory conditions can challenge the stability of Tregs and give rise to proinflammatory Th17 cells (153, 154). This challenge might be resolved by acting directly on muscle Tregs (150, 151), which seem to retain their immunomodulatory potential despite the inflammatory environment, for example by administering low doses of IL-2 (151, 155).

THE ROUTE OF ADMINISTRATION: INTRAMUSCULAR, INTRAVASCULAR, AND SYSTEMIC DELIVERY OF AAV VECTORS TO TARGET THE MUSCLE

Proof-of-concept studies on intramuscular gene transfer with AAV vectors were initially conducted in mice. These studies revealed that the approach has the potential to result in immunity to the transgene encoded by the vector, as evidenced in several studies with vectors encoding for human F.IX (9) and other transgenes (156-158). In some cases, the substitution of strong constitutive promoters with muscle-specific promoters resulted in lower transgene immunogenicity, particularly in the context of muscular dystrophies (157, 158). Scaling up to large animal models of gene transfer confirmed results in mice, showing that intramuscular administration of AAV vectors has the potential of triggering immune responses to the transgene, particularly when large doses of vector are injected in one site (63, 109, 111, 116, 118, 129).

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While immunosuppression has proven efficacious in preventing immunity to the transgene following intramuscular vector delivery (63, 129, 130, 148), the approach has several potential risks and limitations. Additional limitations of the intramuscular vector delivery approach, for example the subtherapeutic levels of transgene expression (67, 77) and the limited vector spread across the muscle, prompted investigators to devise novel vector delivery technologies to transduce large portions to the muscle via the intravascular route (159). The safety and efficacy of this technology has been tested in large animal clotting deficiencies (35), muscle disease (13, 43), and in non-human primates (160-162). Recently the intravascular delivery technology has also been tested in muscular dystrophy patients, demonstrating that the delivery technique is well tolerated (163). From the perspective of immune responses to capsid or transgene, intravascular delivery of AAV to target the muscle exposes the vector to neutralization by pre-existing antibodies (35) and results in systemic exposure to the vector itself, with all possible consequences in terms of activation of T cell responses directed against the capsid (30); furthermore, the delivery technique requires specific skills. Notably, one important advantage of intravascular delivery over intramuscular injection of the vector relies on the fact that it results in dampened transgene immunogenicity (160, 164), which has been associated with induction of protolerogenic T cells in the case of F.IX gene transfer in hemophilia B dogs (164).

In several neuromuscular diseases, the need to target skeletal muscle body-wide and the identification of AAV serotypes able to target muscle following systemic delivery (33, 43, 83, 128), generated broad interest on this route of vector delivery as a one-time treatment solution for diseases affecting skeletal muscle.

Systemic vector delivery, however, results in broad vector biodistribution and targeting of various organs, including the liver, the heart, and, in some cases, the central nervous system. This forces to carefully consider potential toxicities related to immune responses to vector or transgene, and devise intervention strategies to modulate them (31, 32). The use of muscle-specific promoters may help restricting the expression of the transgene to the muscle, thus preventing ectopic expression of the therapeutic protein and potentially reducing its immunogenicity (132). Engineering of chimeric vectors or novel AAV serotypes may also help detargeting tissues in which vector transduction is not desired for therapeutic efficacy (86, 165).

CONCLUSIONS

Muscle has been one of the first tissues targeted with gene transfer with AAV vectors. Over the years efforts have been concentrated on achieving widespread muscle transduction using novel serotypes and delivery techniques. However for muscle diseases requiring whole-body transduction the goal of phenotype correction has not been achieved yet in human trials. Similarly, in several early clinical studies of intramuscular administration of AAV vectors to correct enzyme deficiencies, levels obtained were subtherapeutic (67, 77). Yet, muscle remains a key target for gene therapy, for the treatment of the numerous diseases affecting this organ, and for the fact that it is a valuable target tissues for gene replacement therapy when, for example, it is not possible to target the liver. Importantly, encouraging safety data

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are emerging from muscle gene transfer trials, and evidence of multi-year expression of vector genomes injected into human muscle has been obtained (166).

As the field moves towards systemic approaches of delivery and higher vector doses, it will be essential to keep into careful consideration the interactions between vector and host immune system. In particular anti-AAV antibodies can prevent efficient targeting of the muscle via intravascular and systemic vector administration routes. Novel strategies are being tested to administer the vector in seropositive subjects, and promising results are being obtained, particularly using physical methods to remove antibodies or to block their neutralizing action (52, 53).

Systemic administration of AAV vectors will also likely result in significant spreading of the virus to organs other than the muscle, thus requiring careful monitoring of activation of T cell mediated immunity directed against the capsid or the transgene and potential related toxicities. While data emerging from hemophilia B trials indicate that it is feasible to simply use immunosuppression on demand if liver enzyme elevation is detected (32), this strategy is going to be more difficult to implement in the context of some diseases such as DMD, due to the ongoing disease-related muscle inflammation (167). To this end, a question that remains open, and requires careful risk/benefit evaluation, is whether upfront immunosuppression is warranted, particularly when gene transfer is performed in background of inflamed muscle.

Importantly, from an immunological point of view the muscle is a peculiar environment, where proinflammatory and feedback protolerogenic signals overlap (150, 151). Moving forward towards successful gene transfer in humans it will be key to understand the mechanisms regulating the balance between these two arms of immunity. Devising strategies to skew this balance towards a tolerance state may hold the potential to ameliorating muscle diseases associated with inflammation, allowing for safe and effective gene transfer with AAV vectors.

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Biography

F. Mingozzi

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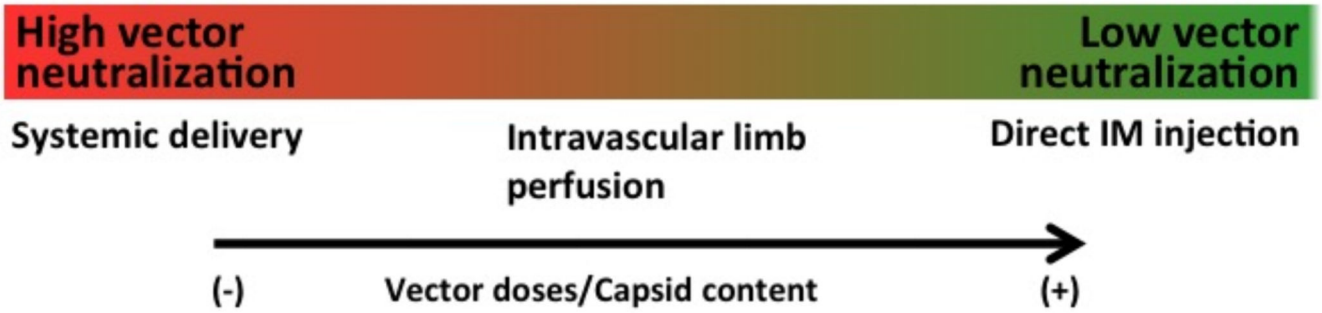

Fig. (1). Route of vector administration and impact of neutralizing antibodies on transduction efficiency

Low-level neutralizing antibodies (NAb) can block target tissue transduction following systemic AAV vector administration. The effect of NAb may be less important when vector is delivered via isolated limb perfusion, which somewhat limits exposure to the systemic circulation. Efficiency of muscle transduction following direct intramuscular (IM) administration of AAV is, conversely, not affected by the presence of NAb. Higher capsid doses have a beneficial effect on transduction efficiency in the presence of NAb to AAV.

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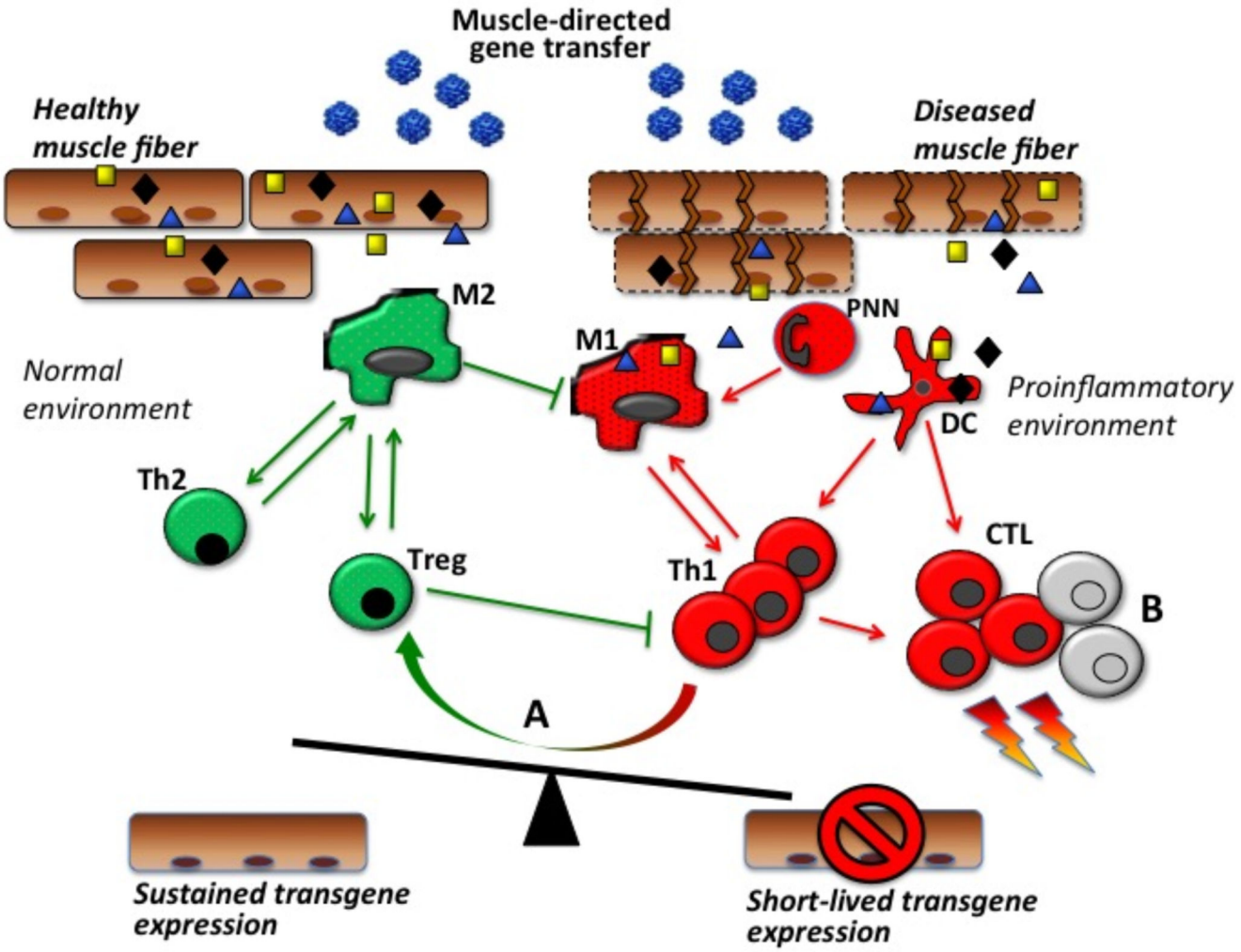

Fig. (2). Transgene expression in muscle and the balance between tolerance and immunity

The release of immunogenic antigens by leaky, damaged muscle fibers upon rAAV intramuscular administration and their uptake by dendritic cells (DC) contribute to the activation of immune responses and destruction of transduced muscle fibers. The proinflammatory conditions provided by the diseased muscle environment drive the activation of effector cells including type 1 macrophages (M1), granulocytes (PNN), type 1 $CD4^+$ T helper cells (Th1), and $CD8^+$ cytotoxic T lymphocytes (CTL). In a normal muscle environment, type 2 macrophages (M2), type 2 $CD4^+$ T helper cells (Th2), and regulatory T cells (Treg) may dampen the effector immune response and contribute to sustained transgene expression. (**A**) in inflamed muscle, resident Tregs may be induced and contribute to modulate immune responses at the muscle level; similarly (**B**) effector T cell exhaustion and/or apoptosis may contribute to limit muscle inflammation.

Table 1
T cell responses to AAV in intramuscular gene transfer trials

Serotype	Transgene	Route	Dose (vg)	Capsid T cell reactivity	Transgene expression in muscle biopsies	Reference (ClinicalTrials.gov ID#)
AAV1	Gamma sarcoglycan	IM	3×10^{9}	0/3	0/3	Herson *et al.*, Brain 2012 (NCT01344798)
			1.5×10^{10}	0/3	0/3	
			4.5×10^{10}	1/3	3/3	
AAV1	Alpha sarcoglycan	IM	3.25×10^{11}	1/3	3/3	Mendell *et al.*, Ann Neurol 2009 (NCT00494195)
			3.25×10^{11}	2/3	2/3	Mendell *et al.*, Ann Neurol 2010 (NCT00494195)
AAV1	Acid-alpha glucosi-dase	IM (diaphragm)	1×10^{12}	0/3 ([H^3] prolif.)	*N/A*	Smith *et al.*, Hum Gene Ther 2013 (NCT00976352)
			5×10^{12}	0/2 ([H^3] prolif.)	*N/A*	
AAV2.5	Minidystrophin	IM	6×10^{11}	1/2 (IS)	0/2	Bowles *et al.*, Mol Ther 2012 Mendell *et al.*, NEJM 2009 (NCT00428935)
			3×10^{12}	0/2	1/2	
			3×10^{12} 6.6×10^{12} capsid particles	0/2 (immunosup-pression, IS)	1/2	
AAV1	Follistatin	IM	2.1×10^{12}	1/3	2/2	Mendell *et al.*, Mol Ther 2015 (NCT01519349)
			4.2×10^{12}	3/3	2/2	
AAV1	Lipoprotein lipase	IM	7.0×10^{12}	2/4	2/3	Mingozzi *et al.*, Blood 2009
			2.1×10^{13}	3/6	3/6	
			2.1×10^{13}	1/4 (IS)	1/2	Ferreira *et al.* , Front immunol 2014 (NCT01109498; NCT00891306)
			7.0×10^{13}	4/13 (IS)	6/8	
AAV1	Alpha-1 Antitrypsin	IM	6.9×10^{12}	N/A	0/3*	Brantly *et al.*, PNAS 2009 (NCT00430768)
			2.2×10^{13}	2/2	Transient*	
			6.0×10^{13}	3/3	3/3*	
			4.2×10^{13}	3/3	1/3*	Flotte *et al.*, Hum Gene Ther 2012 Mueller *et al.* , J Clin Invest 2013 (NCT01054339)
			1.3×10^{14}	3/3	2/3*	
			4.2×10^{14}	3/3	3/3*	

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Table 2
Assays used to measure anti-AAV neutralizing antibodies.

Assay	Advantages	Limitations
Binding antibody assay Capsid particles or pep-tides are coated onto test wells and binding antibodies measured with a secondary anti-Ig antibody	- Easy to setup and validate - Equally sensitive for all AAV serotypes - Allows to measure antibody subclasses - Used in clinical trials to prescreen subjects prior to enrollment	- Binding antibodies are not always neutralizing, which increases the risk of excluding subjects who have no neutralizing antibodies
In vitro cell-based assay The neutralizing antibody titer (NAb) is determined by measuring the residual activity of a reporter vector after incubation with a test sample	- Relatively easy to setup - Used in several clinical trials to prescreen subjects prior to enrollment - Directly reflects the neutralization activity of the sample	- Most serotypes do not efficiently transduce cells *in vitro* - Not very sensitive in detecting low-titer NAb - Validation may be challenging - Uses a reporter vector, not the therapeutic vector, which may have different infectivity
In vivo neutralization assay Residual expression of the vector is measured in mice passively immunized with the test sample	- Works well for most serotypes - Faithfully represents the effect of antibodies on the efficiency of transduction of a target tissue - Has been used in clinical trials to prescreen subjects	- Variability results, need large number of animals - Time consuming and expensive - Difficult to validate

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Table 3
Factors contributing to transgene immunogenicity in AAV gene transfer to muscle

Factor	Increased immunogenicity	Decreased immunogenicity	References
Muscle environment	Inflamed	Normal	(18)
Route of vector administration	Intramuscular	Intravascular	(35, 161, 164)
Vector dose/site (intramuscular)	High dose/site of injection	Low dose/site of injection	(111)
Genetic background	Lack of endogenous protein	Presence of non-functional endogenous protein antigen	(108)
Expression cassette	Constitutive expression cassette	Muscle-specific cassette/detargeting of APCs	(100, 102)
Vector genome conformation	Self-complementary	Single-stranded	(108, 112)
Transgene	Strong T and B cell epitopes		(101)