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Overcoming the Host Immune Response to Adeno-Associated Virus Gene Delivery Vectors: The Race Between Clearance, Tolerance, Neutralization, and Escape

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Abstract

Immune responses in gene therapy with adeno-associated virus (AAV) vectors have been the object of almost two decades of study. Although preclinical models helped to define and predict certain aspects of interactions between the vector and the host immune system, most of our current knowledge has come from clinical trials. These studies have allowed development of effective interventions for modulating immunotoxicities associated with vector administration, resulting in therapeutic advances. However, the road to full understanding and effective modulation of immune responses in gene therapy is still long; the determinants of the balance between tolerance and immunogenicity in AAV vector–mediated gene transfer are not fully understood, and effective solutions for overcoming preexisting neutralizing antibodies are still lacking. However, despite these challenges, the goal of reliably delivering effective gene-based treatments is now in sight.

INTRODUCTION

Gene therapy—the concept of turning genes into medicines by delivering a missing or defective gene to a physiologically relevant target tissue—is one of the most straightforward and compelling concepts in modern biology, yet it has proven challenging to reduce to practice. This is partly related to the fact that it is one of the most complex biologics yet to be developed, consisting typically of both a protein and a nucleic acid component, which must be assembled in a precise fashion for the treatment to be effective. This has required advances in manufacturing, as well as the development of a range of assays for characterization and release of what is essentially a new biologic entity, a recombinant vector particle. Another challenge, which is the subject of the current review, is that vectors that have been derived from naturally occurring viruses have the potential to trigger, and be thwarted by, the human immune response to the parent virus. Further increasing the complexity, the response to a recombinant virion is not the same as the response to a wild-type virus; in the case of adeno-associated virus (AAV) vectors, the recombinant virion can neither replicate nor direct the synthesis of viral proteins, both of which serve to drive the human immune response to the parent virus and are key to the tempo of this response. Thus, it has been critical to elucidate the human immune response to AAV vectors, and to learn how to manage the response, in order to achieve optimal clinical outcomes in patients who receive AAV vectors.

A factor complicating this effort is the hard-won observation that animals imperfectly predict human immune responses to AAV. Although it has been possible to devise animal models of the effects of preexisting antibodies to AAV, it has not been possible to develop robust animal models of the delayed cytolytic T cell responses that were first observed in trials of AAV vectors infused into the liver in men with severe hemophilia (see below). Thus, much of the critical information can be gleaned only from clinical investigation. Finally, many aspects of the human immune response are tissue specific, so that each target tissue of interest presents its own discrete set of problems for solution. Although there are some findings that cut across a variety of tissues, the nuances on which clinical success depends are often tissue specific. This review describes what has been learned thus far, and the areas that require further study.

WILD-TYPE AAV STRUCTURE, FUNCTION, AND HOST RESPONSES

The parent viruses from which AAV vectors are derived are members of the parvovirus family, which consists of small, nonenveloped viruses with a linear single-stranded DNA genome. AAVs are dependoviruses—that is, they require coinfection with a helper virus, either an adenovirus or a herpesvirus, in order to replicate—in fact, they were initially described when they were purified as contaminants of adenovirus in respiratory isolates (1). The viral capsid has an icosahedral configuration, ~26 nm in diameter, and a genome length (for AAV2) of 4,679 bases. The wild-type viral genome encodes two types of proteins: those required for replication (encoded by *rep* genes) and those that serve as viral capsid components (encoded by *cap* genes). An alternative open reading frame that initiates upstream of the *cap* gene encoding VP3 encodes an additional 23-kD protein required for capsid assembly (2). The *rep* and *cap* genes are flanked by two inverted terminal repeats (ITRs) that are required for replication, for packaging into the viral capsid, and for circularization of AAV genomes (3). Recombinant vectors are generated by excising the coding sequences between the two viral ITRs and replacing these with an expression cassette (promoter, transgene, polyadenylation signal) of interest. In a typical manufacturing process, the *rep* and *cap* genes are supplied in *trans*, as is a helper virus (or the necessary genes from the helper virus). A commonly used manufacturing process involves transfection of three plasmids into human embryonic kidney cells (HEK293) (4) or some other human cell line; another uses baculovirus

infection of Sf9 insect cells (5). Both processes yield, in addition to recombinant virions, an excess of empty capsids, and downstream purification processes may or may not remove these. An extensive discussion of manufacturing systems is beyond the scope of this review. Clearly, though, the nature and identity of impurities in the final product, and the likelihood that these contribute to immune responses, are affected by the choice of manufacturing system (6, 7).

Like other viral vectors, AAV vectors capitalize on the normal viral binding and trafficking pathways to gain entry into the target cell. The series of events from vector binding to nuclear trafficking are broadly referred to as transduction, and include binding to cell surface receptors, receptor-mediated endocytosis, vesicular trafficking, endosomal escape, nuclear transport, viral uncoating, and genome conversion (from single-stranded to double-stranded DNA). A number of cell surface molecules have been identified as receptors or coreceptors for AAV; some are capsid serotype–specific, and relative abundance varies by tissue type, accounting at least partly for vector tropism for specific tissues. One of the first identified receptors for AAV2 was heparan sulfate proteoglycan (8). More recently, a protein that appears to be a universal receptor essential for AAV entry into cells, subsequent to cell attachment, has been identified (9). This protein, called AAVR, a predicted type I transmembrane protein with five immunoglobulin-like domains in its ectodomain, shows structural similarity to other well-characterized virus receptors, including those for poliovirus, measles virus, and reovirus. AAVR binds to AAV and traffics from the plasma membrane to the *trans*-Golgi network, and it plays an essential role in the internalization of viral particles, as its genetic ablation renders cells and mice resistant to AAV infection.

Binding to the receptor triggers intracellular signaling pathways that stimulate receptor endocytosis. Endocytic entry not only facilitates bypassing of the cytosol cytoskeletal barriers but may delay immunorecognition as well. The details of endosomal processing are not known but appear to be critical for nuclear transport, because vector injected directly into the cytoplasm fails to accumulate in the nucleus (10). Vector can be retained in the endosomal system for prolonged periods before either escaping into the cytoplasm or being transported to the nucleus. As discussed below, escape of vector into the cytoplasm, followed by proteasomal processing and transport into the endoplasmic reticulum, may result in capsid antigen presentation on the surface of the transduced cell, facilitating detection by circulating immune cells.

Human Immune Response to AAV

In humans, the immune response to recombinant AAV vectors is conditioned by the prior immune response to wild-type AAV. Because wild-type AAV is a dependovirus, and cannot replicate except in the presence of a helper virus, human exposure occurs in the context of infection with a helper virus such as an adenovirus or a herpesvirus. This infection leads to the formation of antibodies to AAV (see below).

In terms of the cellular immune response, although AAV on its own may not induce the significant inflammatory reactions needed for stimulation of a maximal adaptive immune response, in combination with the helper virus, which causes activation of the innate immune system, $CD4^+$ and $CD8^+$ T cells directed to the antigens of the helper virus and of the AAV are formed. Upon control of the infection, the frequency of AAV-specific T cells declines, leaving behind a small pool of memory T cells, which through homeostatic proliferation are maintained throughout life. As a consequence, capsid-reactive T cells can be found in peripheral blood of healthy donors (11, 12), although in a smaller subset of subjects compared with the overall large proportion of humans with circulating antibodies to AAV. To this end, a recent screening of lymphocytes isolated from spleens showed high frequency of subjects bearing capsid-reactive T cells, suggesting that memory AAV-specific T cells do not circulate in peripheral blood at high frequency (13). It is the presence

of these AAV-specific memory T cells that clearly contributes to the differences between the human immune response to AAV vectors and the responses seen in experimental animals.

In the remainder of the review, we summarize aspects of the animal and human immune response to AAV vectors following introduction into different target tissues. The variety of different responses described here underscores the tissue-specific nature of the immune response, which in turn depends upon tissue-specific differences in a whole host of characteristics including the nature and the density of antigen-presenting cells, the cytokine milieu, the existence of anatomic barriers, etc. The immune response may be further modulated by disease-specific alterations of the host immune environment, as occurs, for example, in the setting of Duchenne muscular dystrophy (14).

GENE TRANSFER TO THE EYE

Gene therapy to the eye has enabled ongoing development of therapeutics for classes of disease such as inherited retinal dystrophies that have not previously been treatable with pharmacological therapies. The eye is a highly compartmentalized structure, and efforts have been made to effect gene transfer to both anterior and posterior tissues. The most common delivery routes used for clinical studies thus far have focused on the subretinal space and the vitreous; differences in immune responses between these two target sites illustrate some of the tissue-specific aspects of the immune response to vectors.

Owing to the need to maintain transparency in the structures of the eye, the immune response in the eye has evolved to reduce maximal responses that could result in opacities or lesions. The blood-retina barrier, the avascular nature of much of the eye, and the lack of lymphatics draining the anterior chamber, the vitreous, and the subretinal space all ensure that antigen presentation from the eye is less efficient than from many other organs. Even if these anatomic features are bypassed, immune responses in the eye are characterized by immune deviation. First described in the context of immune responses in the anterior chamber (15), and termed anterior chamber–associated immune deviation, it is now known that antigens introduced to the vitreous or the subretinal space are subject to the same altered response (16). Factors involved in this less robust response, and encompassed in the term anterior chamber–associated immune deviation, include the induction of antigen-specific regulatory T cells (Tregs), the presence of cytokines such as TGFβ2 that contribute to immunosuppression, and a tendency toward Th2 responses, which favor the induction of antibody subclasses that do not fix complement. There are further differences in the immune response in the vitreous compared with that in the subretinal space; the features that characterize these differences are not known with certainty but may reflect differences in the cytokine milieu.

AAV Administration to the Subretinal Space

There is now a decade of clinical experience with AAV administration to the subretinal space (17–19). Vector is administered intraoperatively under direct visualization following a vitrectomy. In line with preclinical studies conducted in large-animal models, most studies have shown an absence of clinical inflammation in the postoperative period, although at least one study (20) observed inflammation at the highest dose injected [1×10^{12} vector genomes (vg)], suggesting that the immune deviation mechanisms can be overcome in the face of high levels of antigen. Most investigators use some form of steroid administration in the perioperative period (17–19) to reduce inflammation and further dampen immune responses.

Biodistribution studies provide some evidence of antigen distribution beyond the retina following subretinal surgery. In the only reported phase 3 study of an AAV vector expressing a gene

required for vision, *RPE65* (21), 13 of 29 subjects (45%) showed detectable DNA sequences in tears, and 10% of subjects showed vector DNA in serum; in most of these cases, tears or sera were negative for vector DNA after one day (Spark Therapeutics, data on file). Overall, transient and low levels of vector DNA were detected in tear and occasional serum samples from 14 of 29 subjects (48%) in the phase 3 study.

To date, the only clinical experience with readministration in the subretinal space occurred in the context of the clinical development program for voretigene neparvovec (21, 22). In the phase 1/2 studies, subjects received a dose ranging from 1.5×10^{10} to 1.5×10^{11} vg in the first eye, followed by a dose of 1.5×10^{11} vg in the second eye administered at time points ranging from 1.7 to 4.6 years after injection of the first eye. There was no evidence of inflammation or deleterious immune responses in response to the first or the second injection, a finding consistent with preclinical studies in affected dogs and nonhuman primates (23). In the phase 3 studies, subjects received a dose of 1.5×10^{11} vg in the first eye, followed by the same dose in the contralateral eye within 12 ± 6 days, again with no evidence of deleterious immune responses. There are no published studies of readministration to the same eye in large animals or in humans.

Intravitreal Administration of AAV

There is less clinical experience with intravitreal administration of AAV, although it has been used in clinical trials for age-related macular degeneration (NCT01024998) and Leber's hereditary optic neuropathy (NCT02064569). Preclinical studies in nonhuman primates by MacLachlan et al. (24) showed the safety of administering a dose of 2.4×10^{9} vg/eye but also showed development of signs consistent with mild to moderate inflammation in 78% of animals at a dose of 2.4×10^{10} vg. Findings were similar in animals injected with an AAV vector not encoding for a transgene, suggesting that the inflammatory response is directed to the vector, not the transgene product. Clinical studies have yielded similar findings, with demonstration of inflammation at doses of 1.8×10^{11} vg in a trial for Leber's hereditary optic neuropathy (25). Safe and efficient transduction of cell types such as retinal ganglion cells, not easily reached from a subretinal site of administration, remains an important goal for intravitreal gene therapy, but results so far suggest that administration of adequate doses is associated with inflammation.

GENE TRANSFER TO THE LIVER

The Liver at the Crossroads Between Tolerance and Immunity

Over the past ~20 years, research efforts have been directed at targeting the liver for the correction of a variety of genetic and metabolic diseases (26). Several important features make the liver an ideal organ for gene therapy, including (*a*) the fact that hepatocytes are central to several metabolic functions and secrete a variety of proteins into the circulation; (*b*) its high degree of vascularization, which allows for easy transduction with any gene therapy vector delivered through the bloodstream; and (*c*) its unique immune environment that is placed at the crossroads between the gut and the bloodstream and therefore has evolved to prevent unwanted responses while preserving the ability to fight infections (27). Evidence of the immune privilege of the liver comes from the transplantation field, as a certain proportion of individuals undergoing orthotopic liver transplantation can completely discontinue antigraft rejection immunosuppression without losing the donated organ (28). Similarly, liver transplantation at an early age followed by immunosuppression exposes children to the development of severe food allergies (29), possibly because of interference with development of peripheral immune tolerance.

Experience with liver gene transfer with AAV vectors reflects the complex nature of this organ when it comes to immune responses. The ability of the liver to accept grafted transgenes and express them in the long term has been established in animal models and humans. The flip side of immunity has also manifested, although only in humans, with what appeared to be a capsid-driven rejection of transduced hepatocytes.

Tolerance to the Transgene Product

In gene transfer, experience with AAV vectors in small- and large-animal models of genetic diseases (30–32) shows that expression of an antigen in hepatocytes can promote robust antigen-specific immune tolerance. Studies with both transgenic animal models (33) and lentiviral vectors (34) further support the validity of this concept.

Extensive characterization of the mechanisms driving liver tolerance has been conducted by several laboratories, and results are sometimes discordant, depending on the experimental setting and antigen model. Induction of antigen-specific Tregs has been documented in several studies (30–32, 35), together with apoptosis of reactive T cells (31), induction of $CD8^+$ Tregs (36), and other mechanisms (37). The crucial role of Tregs in liver-mediated tolerance induction was confirmed in small- and large-animal models of AAV vector–mediated gene transfer, in which pharmacological blockade or depletion of Tregs around the time of vector administration resulted in an immune response against the transgene (30, 32). In particular, in nonhuman primates, we showed that administration of the anti-CD25 monoclonal antibody daclizumab at the time of AAV vector administration led to the development of antibodies against the secreted transgene, which was the result of the transient blockade of Treg induction (30).

Although preclinical data on liver tolerance are highly convincing, the open question is whether this concept will faithfully translate to humans. Thus far, published clinical studies of liver gene transfer have been conducted only in the context of hemophilia B and acute intermittent porphyria trials (38–41). None of the subjects enrolled in the AAV hemophilia B trials developed immune responses against the donated therapeutic transgene, despite the fact that some participants had null mutations and therefore were cross-reactive immunological material (CRIM) negative (39–41). However, it should be noted that all subjects enrolled had extensive exposure to protein replacement therapy before gene therapy, and those who had a history of inhibitor formation were excluded from enrollment. Thus, results to date may underestimate the true risk of developing an immune response to the transgene product following liver-directed gene transfer in patients previously untreated with protein replacement therapy. Future studies will help to address this point. Liver gene transfer, compared with protein replacement therapy, is likely to be less immunogenic from a transgene perspective, although the interactions of AAV vectors with the innate immune system influence the outcome of gene transfer. For example, the AAV vector DNA genome and its capsid are sensed by TLR9 (42–45) and TLR2 (46), respectively, resulting in potentially higher immunogenicity of vectors, particularly for AAV vectors carrying double-stranded rather than single-stranded genomes (45).

Cellular Immune Responses to Capsid Following Gene Transfer to the Liver

Although it is well established that animal studies are imperfect predictors of side effect profiles in humans, it nonetheless came as a surprise when the first liver trial of AAV exhibited a series of findings that had not been seen in any animal models, including mice, hemophilic dogs, and nonhuman primates (**Figure 1*a***) (39). Specifically, approximately 4 weeks after vector infusion, the liver transaminases (alanine aminotransferase, aspartate aminotransferase) began to rise, peaking at about 6 weeks and gradually returning to normal levels over the ensuing 8 weeks without any

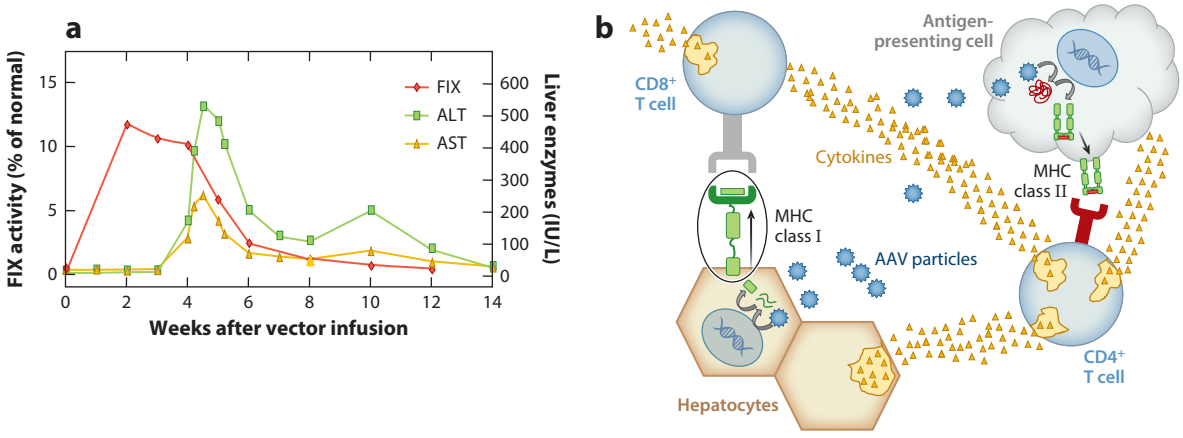


Figure 1

Cellular immune responses to the AAV capsid following gene transfer to the liver. (*a*) Subject E in the first AAV-FIX liver trial exhibits therapeutic levels of FIX (10–12%) for 4 weeks, and then FIX levels gradually decline, accompanied by an asymptomatic and self-limited transaminase elevation. Adapted from Reference 11. (*b*) Working hypothesis to explain loss of FIX and transaminase elevation. AAV particles enter the target hepatocyte. Some particles remain in the cytosol and are broken down into peptides, and the peptides are presented via MHC class I molecules on the surface of the transduced cell, making it a target for circulating capsid-specific $CD8^+$ T cells. Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; FIX, factor IX; MHC, major histocompatibility complex.

medical intervention. In tandem, the circulating factor IX (FIX) levels, which had been stable in the range of 10–12% of normal for the first 4 weeks after vector infusion, also slowly declined and by 12 weeks after vector infusion were again at <1%. The subject, a 31-year-old male, remained asymptomatic throughout this time and at the conclusion retained his ability to respond well to infused clotting factor concentrates, with unchanged kinetics. The investigators considered a range of etiologies for the asymptomatic, self-limited rise in transaminases, but most were quickly excluded on the basis of laboratory testing, and the kinetics were highly suggestive of an immune response. The protocol was modified to include more extensive immunomonitoring, and data gathered from a subsequent subject who exhibited similar findings demonstrated that (*a*) based on enzyme-linked immunospot data, a population of capsid-specific $CD8^+$ T cells expands and then contracts following vector infusion; (*b*) the kinetics of the expansion and contraction parallel the transaminase rise and fall; and (*c*) the expanded $CD8^+$ T cells specifically lyse human leukocyte antigen (HLA)-matched, AAV-transduced target cells. Moreover, studies in normal subjects demonstrated the presence of circulating capsid-specific memory $CD8^+$ T cells in rare individuals (11).

This led the investigators to propose a working model in which the transaminase elevation reflected the specific destruction of the transduced hepatocytes by capsid-directed $CD8^+$ T cells (**Figure 1*b***). Further support for this hypothesis came from mapping the capsid-specific epitopes by enzyme-linked immunospot and demonstrating that these peptides were predicted to bind with high affinity to one of the subject's major histocompatibility complex (MHC) class I molecules. The basis of capsid antigen presentation by the transduced hepatocytes was hypothesized to be the induction into class I pathways of capsid remaining in the cytosol. Previous work had shown that capsid proteins undergo proteasomal processing (47, 48). The capsid-derived peptides, upon transport to the endoplasmic reticulum, are complexed with MHC class I molecules and presented on the surface of the transduced cell (49). It was proposed that this sufficed for recognition of AAV-transduced cells by $CD8^+$ effector T cells. This raised the question of why

Table 1 Hypotheses to explain transaminase elevation and loss of FIX expression after vector infusion to the liver in humans with hemophilia B

Underlying cause	Prediction	Reference(s)
Preformed capsid inducted into class I presentation pathways in transduced hepatocytes	Short-term IS will work	39, 40
Rep/cap plasmid has been packaged	Short-term IS will not work	52
Alternate ORFs	Short-term IS will not work	54
Heparan sulfate–binding site on AAV2 capsid results in efficient transduction of antigen-presenting cells	AAV8 will not trigger immune response	50

Abbreviations: FIX, factor X; IS, immunosuppression; ORF, open reading frame.

other animal models had not manifested similar findings, but humans' prior exposure to AAV capsid and the presence of memory T cells likely underlie this difference. Because memory T cells are more readily triggered than naive T cells, humans, undergoing reexposure, have a different outcome than experimental animals do. Given the fact that the vector does not direct the synthesis of capsid antigen, which should be present only transiently, the investigators proposed that a short immunomodulatory regimen might suffice to block the immune response until the capsid-derived peptides had been cleared from the cell.

A number of other hypotheses were subsequently advanced to explain the findings (**Table 1**). Vandenberghe et al. (50) proposed that a specific motif (the heparan sulfate–binding domain) in the AAV2 capsid favors receptor-mediated uptake of vector by human antigen-presenting cells, with activation of capsid-specific $CD8^+$ T cells occurring on that basis. They proposed that the use of alternate capsids, such as AAV8, that lack this motif would not be associated with an immune response. Against this was that the peptide epitope that had been identified in the clinical trial described above was highly conserved in multiple capsid serotypes, including AAV8, and in addition, peripheral blood mononuclear cells expanded with whole capsids of AAV2 cross-reacted when presented with capsid from AAV8. In a subsequent liver-directed clinical trial (40, 41), AAV8 triggered a similar series of findings (transaminase elevation, loss of FIX expression, and expansion of capsid-specific T cells) at the same dose, suggesting that this theory was incorrect. Moreover, this trial had incorporated a provision to administer a short course of steroids if these findings were identified. The steroids resulted in rapid disappearance of the capsid-reactive T cells from the circulation, resulting in long-term expression of the transgene. Subsequent studies showed that the capsid-reactive T cells reappeared in the circulation after the steroids were stopped but were not associated with any clinical sequelae, presumably because capsid-derived peptides had been cleared from the transduced hepatocytes so that the targets were no longer detected by circulating lymphocytes.

Other hypotheses advanced after the first trial included the possibility that plasmid expressing the capsid gene had been packaged into the recombinant virions, so that capsid proteins were being continuously expressed and the immune response arose on that basis (**Table 1**). Encapsidation of prokaryotic sequences from plasmids used in production has been documented (51), but careful study of the clinical lots of vector used in the first trial showed no evidence of capsid expression in cell lines transduced at high multiplicities of infection (MOIs), or in mice injected with the vector (52). One would predict that if this had been the case, steroids would not have controlled the immune response, because capsid proteins would be continuously expressed and the immune response would reappear once steroids were stopped. An excellent response to steroids was observed in the trials reported by Nathwani et al. (40, 41), and in the more recent trials reported by George et al. (53), making this explanation less likely. Yet another hypothesis focused on the possibility that alternate open reading frames in the expression cassette were leading to production

of neoepitopes (54). Again, in this case, short-term immunosuppression should be ineffective at rescuing expression, as the alternate open reading frames should be expressed continuously until all transduced cells are destroyed, at which point all transgene expression from the donated gene should also be gone. Thus, the now demonstrated ability to rescue expression in clinical studies with transient immunomodulatory regimens (40, 53) makes this hypothesis unlikely.

It should be noted that the inability to develop an animal model that recapitulates the series of findings in humans not only has hampered efforts to address the deleterious immune response in clinical studies but also accounts at least in part for the skepticism that surrounded the initially proposed basis of the cellular immune response—that is, induction of input capsid into MHC class I presentation pathways. In early studies after the first report of the findings, mice were immunized with AAV capsid and then injected with AAV vectors, but no diminution of FIX expression was observed (55–58). Efforts to boost the immune response, including the use of adjuvants (59) or incorporation of the SIINFEKL epitope into the AAV capsid coupled with use of OT-1 mice (55, 60), recapitulated many of the features of the human immune response, but the physiological explanation of the need to boost the response in mice was elusive, which raised questions regarding the relevance of these models. Moreover, even in nonhuman primates, which have naturally displayed preexisting immunity to AAV, it was not possible to observe similar findings. Previous work by Varki and colleagues (61) had proposed that the loss of an inhibitory immunoglobulin-like lectin on the surface of T cells during evolution from nonhuman primates to humans might account for the lower threshold for T cell activation in humans compared with nonhuman primates. Subsequently, Ertl and colleagues (62), using intracellular cytokine staining combined with staining for T cell subset and differentiation markers to study both human and rhesus capsid-specific $CD8^+$ T cells, documented striking differences in the differentiation status and functional activities of the cells, providing a possible explanation for why human findings have not been modeled in other species.

In contrast to the perplexities surrounding the attempts to develop animal models, the data generated from the study of human cells with specialized reagents have been much more straightforward to interpret. The investigators involved in the original AAV liver trial cloned the complementary DNA sequence encoding the T cell receptor (TCR) that recognized the epitope described in the early work and used this to generate a T cell line that stably expressed the TCR and thus recognized the peptide-MHC complex of interest (VPQYGYLTL presented by HLA-B*0702) (49, 63). The T cell line was devoid of endogenous TCR β chain and had been engineered to express luciferase in proportion to the engagement of the donated TCR (64). Transduction of an HLA-matched human hepatocyte line at increasing MOIs resulted in a dose-dependent increase in the luciferase signal from the T cells, reflecting an increase in antigen presentation on the hepatocytes with increasing MOIs (**Figure 2**). Moreover, the investigators showed that treatment of the hepatocytes with the proteasomal inhibitor bortezomib could, in a dose-dependent manner, extinguish the luciferase signal in the cognate T cells and could also inhibit killing in a cytotoxic T lymphocyte assay, suggesting a potential therapeutic approach to the human $CD8^+$ T cell response to capsid (49). The greater value of this setup, however, is that it provides an experimental system for testing pharmacological interventions to prevent or modulate the human $CD8^+$ T cell response.

Additional studies that occurred after the first liver-directed AAV trial also highlighted the role of both the DNA conformation (single stranded versus double stranded, CpG minimized or not) (45, 65) and the capsid (46) in the innate immune response, as they are sensed by TLR9 and TLR2. This innate immune response in turn affects the subsequent adaptive immune response. Differences in some or all of these elements of the vector may account for the range of clinical results observed in recently reported trials of AAV-mediated, liver-directed gene therapy (**Table 2**): Some vectors exhibit excellent control of immune responses with adjuvant steroids, whereas others are resistant to this intervention.

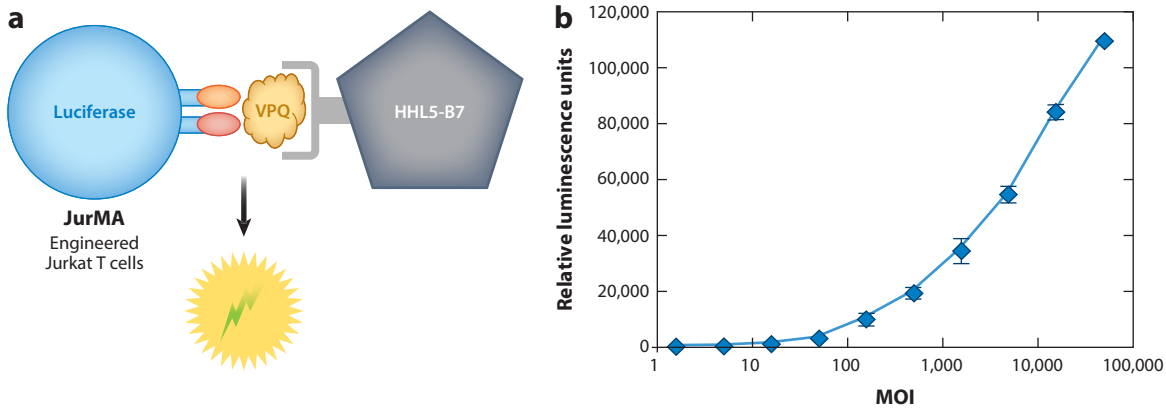


Figure 2

Detection of capsid antigen presentation onto major histocompatibility complex (MHC) class I. (*a*) HHL5-B7 liver cell line expressing human leukocyte antigen (HLA)-B*07 transduced with AAV vectors (*right*) presents capsid-derived peptides (VPQ) on the cell surface. AAV capsid–specific JurMA-VPQ reporter T cell line (*left*) expresses luciferase in proportion to engagement of its T cell receptor. (*b*) Detection of luciferase expression from engineered JurMA T cells as a function of increasing multiplicity of infection (MOI) of the target hepatocyte cell line. Adapted from Reference 49.

As was perhaps predictable from the early clinical results, which established that animal models did not predict the existence of this $CD8^+$ T cell response to capsid-transduced hepatocytes, most of the field's learnings about this response have come from careful studies of the immune response in clinical trials. Though few of the trials have been the subject of full-length publications at this point, there are some clear conclusions from the published data. First, the response is dose dependent, so efforts to achieve better efficacy with lower doses are useful. Second, vectors evaluated in clinical studies to date differ in terms of whether immune responses associated with their administration can be effectively controlled with steroids. There is not agreement on which if any properties of the vector may account for these differences, but candidates include DNA conformation (45), presence of CpG dinucleotides in the DNA (65), vector capsid identity (50), content and amount of product-related impurities (51, 52), and content of empty capsid in the final formulation (66).

GENE TRANSFER TO THE CENTRAL NERVOUS SYSTEM

Together with the eye and the liver, the brain has been historically defined as an immune-privileged body site. Although the organ is somewhat isolated from the general circulation by the blood-brain barrier (BBB), experience with autoimmune diseases affecting the central nervous system (CNS) (67) would argue that this organ is not completely protected from inflammation. This is well illustrated by studies in which immunotherapy for glioblastoma has been tested (for a review, see 68), together with the evidence that antigen-presenting cells (69) and cytotoxic T cells (70) can cross the BBB.

Together with lentiviral vectors in ex vivo gene transfer (71, 72), AAV vectors have been regarded as the tools of choice for the development of CNS gene therapy approaches because of their ability to transduce widespread areas of the brain, to target neurons, and to drive stable long-term expression of a donated gene.

To date, several in vivo trials with AAV vectors for diseases affecting the brain or motor neurons have been performed (reviewed in 73). As for other indications, early studies were mainly

Table 2 Results from AAV-mediated, liver-directed hemophilia trials

Sponsor(s)	Capsid	Transgene	Result	Reference(s)
Avigen	AAV2	Wild-type FIX single-stranded cDNA	Transient expression of FIX level 10–12% at dose of 2×10^{12} vg/kg in one of two subjects who received this dose.	39
University College London and St. Jude Children's Research Hospital (NCT00979238)	AAV8	Codon-optimized wild-type FIX self-complementary cDNA	Long-term expression of 2.9–7.2% (average 5.1%) at dose of 2×10^{12} vg/kg. Four of six subjects required short course of steroids at this dose.	40, 41
Baxalta/Shire (NCT01687608)	AAV8	Codon-optimized FIX Padua self-complementary cDNA	One of eight subjects achieved long-term expression at level ~20%. Most others expressed therapeutic levels initially but lost expression despite course of steroids. No immune response to FIX Padua.	130
Spark Therapeutics and Pfizer (NCT02484092)	Novel bioengineered capsid	Codon-optimized FIX Padua single-stranded cDNA	Ten of ten subjects achieved long-term expression at levels >12%, with average plateau level >28%. No immune response to FIX Padua. Two of ten subjects required short course of steroids.	53
uniQure (NCT02396342)	AAV5	Codon-optimized wild-type FIX self-complementary cDNA	Zero of ten subjects achieved levels >12%. Average levels in four of five subjects in low-dose cohort (5×10^{12} vg/kg) 5.4%. One subject failed to express. Average levels in second dose cohort (2×10^{13} vg/kg) 7%. Two of ten subjects treated with short course of steroids.	131
Dimension Therapeutics (NCT02618915, NCT02971969)	AAVrh10	Codon-optimized wild-type FIX self-complementary cDNA	All patients had evidence of transgene expression (observation ongoing). Five of six patients experienced ALT elevation, peaking at 914 IU/L in one subject from the high-dose cohort (3.5×10^{12} gc/kg).	132 (press release)
BioMarin (NCT02576795)	AAV5	BDD wild-type FVIII cDNA	Nine subjects treated. Seven of seven subjects treated at 6×10^{13} vg/kg expressed FVIII at levels ranging from 10% to >200%. Steroids administered to all subjects from the high-dose cohort.	133

Abbreviations: ALT, alanine transaminase; BDD, B domain deleted; cDNA, complementary DNA; FVIII, factor VIII; FIX, factor IX; gc, genome copies; vg, vector genomes.

conducted with AAV2 vectors, due to the fact that this serotype was among the first to be isolated and characterized. Doses used were generally low, and vector was delivered focally or intraparenchymally. Among these initial studies are ones for Parkinson's disease (74), Canavan disease (75, 76), and Batten disease (77). In all these studies, vector delivery was well tolerated, and at the systemic level no significant capsid-driven immune responses were detected, except for a modest increase in anti-AAV neutralizing antibodies (NAbs) in blood observed after vector administration. Similar results were also obtained in a recent trial of gene therapy for aromatic L-amino acid

decarboxylase (AADC) deficiency, in which an AAV2 vector encoding the AADC transgene was delivered to the putamen of patients at a dose of 1.6×10^{11} vg, resulting in improvement of motor function (78).

Intraparenchymal delivery of AAV vectors has also been proposed for the treatment of mucopolysaccharidosis (MPS) types IIIA (an AAVrh10 vector) and IIIB (an AAV5 vector) (73). In these clinical studies, vectors were administered directly into the brain parenchyma via catheters inserted into burr holes in the skull. No adverse events associated with vector or transgene immunogenicity were reported, although complete results of immunomonitoring studies are not published yet. In these two trials, immunosuppression was given together with the vector based on preclinical results in affected dogs showing high levels of inflammation in the brain after gene transfer with vectors encoding the human transgene (79).

Whether transgene immunity is a true concern in the setting of gene transfer directed to the CNS remains to be fully established. Important questions remain to be answered—for example, whether the route of administration will affect the immune responses against the transgene. In the setting of brain gene transfer, preclinical studies conducted by Haurigot et al. (80) in the setting of intracerebroventricular gene transfer for MPS IIIA, an approach that results in uniform transduction across the CNS, including neurons and ependymal cells, showed no immunogenicity of the canine transgene. Conversely, animals had a robust inflammatory response to the human transgene, suggesting that the species-specific transgene delivered intracerebroventricularly is likely to be poorly immunogenic. One caveat in the interpretation of these results is the fact that these experiments were conducted in wild-type dogs, so animals were tolerant to the transgene product.

In the clinical setting, and thus in the setting of a defective gene, it will be extremely important to monitor transgene immune responses and to correlate findings with the CRIM status of the recipients (i.e., CRIM-positive patients have a theoretically lower risk of developing an immune response against the transgene). Immunosuppression may ultimately be required to guarantee transgene acceptance in some patients (i.e., CRIM-negative patients), and measures such as the use of AAV vectors (81) or delivery routes that are more likely to result in widespread brain transduction (80) may help reduce the risk of transgene immunogenicity. Ongoing and planned clinical trials of AAV gene transfer for MPS IIIA in which the therapeutic vectors are delivered intraparenchymally via burr holes (75, 76), intracerebroventricularly (80), or intravenously (82) will help elucidate the critical parameters for successful CNS gene transfer for lysosomal storage diseases. Additional studies in which the vector is delivered intrathecally are ongoing (74) and will also yield new information on immune responses in this route of administration.

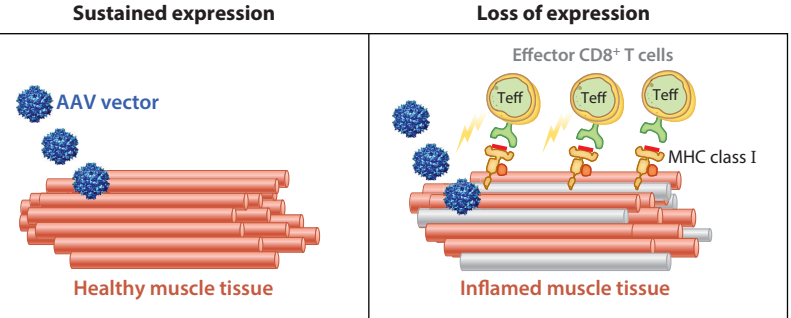
With the identification of AAV vectors able to cross the BBB (83, 84), and modifications to the AAV capsid that enable specific targeting of the brain parenchyma (85), systemic delivery of AAV vectors to target motor neurons and the CNS has been proposed. In one ongoing study of gene therapy for spinal muscular atrophy (NCT02122952), an AAV9 vector was infused at doses $>10^{14}$ vg/kg into infants. Vector infusion was generally well tolerated; however, about 2 weeks after vector administration, an increase in liver enzymes was observed. Concomitantly, capsid-specific T cells were detected in peripheral blood. As for hemophilia B (40, 41), a course of corticosteroids given prophylactically seemed to prevent the immune response. The role of the age of the subjects, both in terms of immunological naivety to wild-type AAV and lack of a fully mature immune system, in the outcome of this trial is unknown.

In conclusion, results obtained thus far suggest that the disease and the requirement for the widespread transduction of the CNS or for targeting of motor neurons can drive the choice of the serotype or the route of vector delivery. Small doses delivered intraparenchymally do not lead to major systemic exposure to the vector. Conversely, with increasing vector doses delivered

intraparenchymally, or into the cerebrospinal fluid or the bloodstream, systemic exposure to the vector is significant. Along this scale of increasing exposure to the vector, the risk of experiencing immunotoxicities is likely to increase. For systemic delivery of AAV, the presence of preexisting antibodies to the vector needs to be taken into account (see below).

MUSCLE GENE TRANSFER

The muscle is a key target for gene therapy for the treatment of the numerous diseases affecting this organ (87–97) and can also be a valuable target for production of therapeutic proteins when, for example, it is not possible to target the liver (89, 94, 95, 98–100). Encouraging data are emerging from muscle gene transfer trials (101, 102), and evidence of multiyear expression of vector genomes injected into human muscle has been obtained (103). So far, clinical trials of muscle gene transfer have mostly been conducted by administering the vector intramuscularly, mainly to assess the safety of the approach. Due to the systemic nature of most neuromuscular diseases, current efforts are aimed at more systemic approaches, in which large doses of AAV vectors are administered systemically (104, 105). Although preclinical results in large-animal models demonstrated that whole-body correction of neuromuscular diseases is feasible with AAV-mediated gene transfer, targeting the muscle with AAV-mediated gene transfer poses some potential constraints related to immune responses and their determinants (**Figure 3**). Among the factors that can shape the outcome of gene transfer in muscle, the local immune environment is one of the most important, as several neuromuscular disorders are associated with muscle inflammation (14) or an altered immune system. Thus, in addition to the vast heterogeneity in the disease state of muscle in neuromuscular disorders, the presence of inflammation may affect the context in which either the vector or the encoded transgene will be presented to the immune system (106), for example by upregulating MHC class I expression. This point is clearly exemplified by Duchenne muscular dystrophy, a disease in which preexisting immunity to the dystrophin protein, likely deriving



Muscle environment	Normal	Inflamed
Route of delivery	Intravascular	Intramuscular
Genetic background	Presence of nonfunctional endogenous protein	Complete lack of endogenous protein
Expression cassette	Muscle specific or detargeted from antigen-presenting cells	Constitutive expression cassette
AAV vector genome	Single-stranded	Self-complementary

Figure 3

Determinants of transgene immunogenicity in muscle gene transfer. Abbreviation: MHC, major histocompatibility complex; Teff, effector T cell.

from revertant fibers, was documented in untreated patients (14). The impact of this finding on the safety and efficacy of gene transfer remains to be fully established, and the only evidence that preexisting immunity to dystrophin can have an impact on the stability of gene transfer comes from a clinical trial in which an AAV vector expressing a truncated form of dystrophin was administered intramuscularly to patients (107).

Second to the local immune environment, the route of vector delivery plays a central role in muscle gene transfer with AAV vectors as a determinant of transgene immunogenicity, as demonstrated by animal studies showing that intramuscular vector delivery results in more pronounced immune responses compared with intravascular (108, 109) or systemic (104, 105) vector delivery. This could be partly due to the fact that intravenous vector delivery results in a more uniform and widespread transduction of muscle, whereas intramuscular delivery concentrates the transgene production mainly at the site of injection. To this end, preclinical results in the context of hemophilia B gene transfer with AAV vectors clearly demonstrate that the amount of local antigen expressed in the muscle is a major determinant of transgene immunogenicity (110). In addition to genetic background and local vector dose, recent studies in mice showed that self-complementary AAV vectors encoding FIX injected intramuscularly can lead to a stronger $CD8^+$ T cell response to the transgene compared with single-stranded vectors (111, 112), a result in agreement with previous findings in the context of AAV gene transfer to the liver (45).

Capsid-Directed Immune Responses in Muscle

As for liver, in intramuscular gene transfer clinical trials, the emergence of cytotoxic T lymphocytes against the AAV capsid after intramuscular administration of AAV vectors 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 clinical trials, T cell reactivity to the AAV capsid has also been associated with an apparent lack of transgene expression (106, 113). This has prompted investigators to implement protocols in which immunosuppression is given concomitant with vector administration (114–116). Despite the detection of T cell reactivity against the AAV capsid in peripheral blood mononuclear cells, and the identification of infiltrates in the muscle of AAV-treated patients, sustained transgene expression has been documented in subjects who received an AAV1 vector encoding α1-antitrypsin injected intramuscularly (101, 117, 118). Interestingly, $CD4^+CD25^+FoxP3^+$ Tregs were also found within the infiltrating cells in vector-injected muscle, which may account for the control of capsid cytotoxic T lymphocyte responses locally (101). 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. However, despite the apparent control of immune responses mediated by regulatory mechanisms, recent work in the context of the same α1-antitrypsin trial suggests that in some patients a transgene-specific cytotoxic T cell response, driven by antigen presentation in the context of a rare HLA-C allele, was responsible for the partial loss of transgene expression (119).

Treg infiltrates were also found in a study of AAV1 muscle gene transfer for lipoprotein lipase deficiency; however, the administration of an intensive immunosuppressive regimen in this study may also have affected the outcome of gene transfer (115, 116). Nevertheless, data emerging from preclinical (120) and clinical (106) 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.

Although targeting neuromuscular disorders systemically with AAV vectors has been the object of intense investigation, in some cases leading to promising results (103, 104), so far this approach

has been tested in the clinic only to treat young children affected by spinal muscular atrophy. One of the main reasons for this delay has been the need to scale up AAV vector production to allow for vector administration to subjects at vector doses exceeding 10^{13} vg/kg (103, 104).

From a therapeutic point of view, moving from local delivery of AAV vectors to more systemic approaches for several muscle diseases seems reasonable, but from an immunological point of view, this shift will change the vector biodistribution and likely the overall nature of the response. The spinal muscular atrophy trial of AAV9 gene transfer for *SMN1* is an example of this point (see above), as systemic vector administration to target motor neurons resulted in off-target immunotoxicities in the liver.

NEUTRALIZING ANTIBODIES TO AAV

Exposure to wild-type AAV results in a significant proportion of individuals carrying humoral immunity against the capsid, which becomes detectable early in life, beginning around 2 years of age (121–123). Due to the high degree of amino acid sequence conservation among AAVs (124), anti-AAV antibodies show cross-reactivity with a wide range of natural (125) and engineered serotypes. While preexisting NAbs to AAV are not an issue for gene transfer applications targeting the eye, or when the vector is administered directly into the parenchyma, they pose an important limitation on the use of AAV vectors in the clinic whenever vector is delivered through the circulation (e.g., to target the liver).

The influence of NAbs on the ability to transduce the liver with AAV vectors became obvious in the first trial in which an AAV vector was introduced through the circulation to target the liver. A marked difference in initial levels of FIX expression in two individuals who both received a dose of 2×10^{12} vg/kg, but who differed in terms of pretreatment NAb titer to AAV, was noted (39). The subject with a low pretreatment NAb titer manifested a peak level of FIX expression of 12% of normal, whereas the subject with a pretreatment NAb titer of 1:17 did not show detectable expression from the transgene. Subsequent dose-response studies in mice and nonhuman primates (126, 127) demonstrated that NAb titers $\geq$1:5 effectively block transduction when vector is delivered intravenously. Most trials in which vector is delivered through the circulation now prescreen potential subjects with an NAb assay and exclude those with titers above some specified cutoff value. Sponsors and investigators use a variety of different NAb assays (128); typically, they involve transduction of a cell line with a reporter gene in the presence and absence of serum from a subject, but assays vary widely in the reporter gene, the serum incubation conditions, the cell lines, etc., so that it is difficult to compare results from one group to those of another.

Today, seropositive subjects are excluded from most gene therapy trials. Additionally, following vector administration, NAbs are induced at high titers (39, 129), preventing vector readministration (**Table 3**). Clearly, though, it is important to develop solutions that, alone or in combination, will allow some or all individuals bearing circulating antibodies to AAV to be successfully treated and to allow for redosing AAV vectors if needed (**Table 4**).

Table 3 Persistence and cross-reactivity of anti-AAV2 neutralizing antibodies (NAbs) following vector administration

Subject ID	Baseline AAV2 NAb titer (reciprocal dilution)	Follow-up (years)	AAV2 NAb titer (reciprocal dilution)	AAV8 NAb titer (reciprocal dilution)
A	1:2	9	>1:3,160	1:1,000
B	1:11	9	1:3,160	1:1,000
C	1:2	7	>1:3,160	1:100
D	<1:2	2	>1:3,160	1:100

Table 4 Strategies to overcome neutralizing antibodies (NAbs) to AAV vectors

Strategy	Pros	Cons	Clinical feasibility
Select patients with low or no NAbs	■ No need for intervention ■ Simple to implement (128)	■ Can result in exclusion of several candidates (125)	Currently broadly adopted in gene therapy trials
Use less-seroprevalent capsids or switch serotype	■ No need for pharmacological intervention	■ Almost all serotypes are cross-neutralized (125) ■ Each new serotype is a new product to be developed	Hard to implement due to the high costs associated with bringing multiple serotypes to the clinic
Plasmapheresis (134, 135)	■ Safe and effective in reducing antibody titers ■ Proof-of-concept studies in monkeys and humans promising	■ Requires multiple cycles of plasma absorption ■ Less efficient with high-titer NAbs ■ Nonspecific, depletes all immunoglobulins	Likely feasible, technology already available in hospitals
Immunosuppression	■ Some technologies seem promising (136–138)	■ Most drugs ineffective at eradicating antibodies (138) ■ Global immunosuppression associated with side effects and can interfere with gene transfer (30, 139)	Feasible, granted a favorable risk/benefit ratio; most likely effective in the prevention setting (to allow for vector readministration) (140)
Isolated organ perfusion	■ Proof-of-concept results promising in liver gene transfer (141) ■ Does not require immunosuppression	■ Does not work well in the presence of high-titer NAbs ■ Not useful in the setting of systemic diseases	Procedure not currently in use in the clinic; invasive
Increase the capsid dose or use capsid decoys	■ Proof-of-concept results promising in liver gene transfer (66) ■ Does not require immunosuppression	■ Higher vector doses may pose a constraint in terms of manufacturing ■ Unlikely to be effective with NAb titers >1:100 (66)	Feasible, but may contribute to vector antigen load

SUMMARY POINTS

1. AAV vector–mediated gene transfer has resulted in long-term therapeutic efficacy in humans affected by a variety of diseases. However, preclinical and clinical experience indicates that components of AAV vectors can be recognized by the host immune system.
2. Thus far, no serious or permanent consequences of immune responses, other than a transient, asymptomatic elevation of liver enzymes, have resulted from AAV vector administration in humans, reflecting the poorly inflammatory profile of these vectors.
3. Depending on the tissue targeted with gene transfer, and the level of systemic exposure to the vector, humoral and cell-mediated immune responses directed against the AAV capsid can differ dramatically. For example, low doses of vector delivered subretinally are generally well tolerated and are not associated with significant activation of the immune system.

4. Conversely, in liver gene transfer, success in modulating vector immunogenicity has been a crucial determinant of the ability to achieve sustained and consistent therapeutic efficacy in patients.
5. Humoral immunity to AAV, generated in the context of exposure to the wild-type vector or triggered by AAV vector administration, prevents effective gene transfer when the vector is delivered systemically. Several solutions have been proposed and hopefully will be translated to the clinic in the near future.

FUTURE ISSUES

1. How are empty capsids processed and presented, and are processing and presentation of empty capsids identical to the corresponding pathways for full capsids?
2. Why are some capsid-directed $CD8^+$ T cell responses controlled with steroids and others not?
3. Why do some subjects experience immune-mediated toxicities after gene transfer and others not?
4. What is the role of innate immunity when it comes to immune responses to AAV vectors in humans?
5. Is transgene immunogenicity going to be an obstacle in the clinical translation of some AAV-based gene therapies?
6. What are the most effective methods for overcoming or circumventing preexisting antibodies to AAV? Will there be different methods depending on the titer of preexisting NAb?
7. What are the most effective methods for preventing formation of antibodies following AAV vector administration? Can we safely and efficiently readminister AAV vectors?

DISCLOSURE STATEMENT

F.M. has consulted for companies developing AAV vector–based technologies and is an inventor on patents describing the AAV vector technology. K.A.H. is an employee of and holds equity in Spark Therapeutics.

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