Molecular Therapy
Review

AAV Vector Immunogenicity in Humans: A Long Journey to Successful Gene Transfer

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Gene therapy with adeno-associated virus (AAV) vectors has demonstrated safety and long-term efficacy in a number of trials across target organs, including eye, liver, skeletal muscle, and the central nervous system. Since the initial evidence that AAV vectors can elicit capsid T cell responses in humans, which can affect the duration of transgene expression, much progress has been made in understanding and modulating AAV vector immunogenicity. It is now well established that exposure to wild-type AAV results in priming of the immune system against the virus, with development of both humoral and T cell immunity. Aside from the neutralizing effect of antibodies, the impact of pre-existing immunity to AAV on gene transfer is still poorly understood. Herein, we review data emerging from clinical trials across a broad range of gene therapy applications. Common features of immune responses to AAV can be found, suggesting, for example, that vector immunogenicity is dose-dependent, and that innate immunity plays an important role in the outcome of gene transfer. A range of host-specific factors are also likely to be important, and a comprehensive understanding of the mechanisms driving AAV vector immunogenicity in humans will be key to unlocking the full potential of *in vivo* gene therapy.

INTRODUCTION

Recombinant adeno-associated virus (AAV) vectors are derived from small, non-enveloped, 4.7-kb DNA dependoviruses belonging to the Parvoviridae family. During the past several years, *in vivo* gene therapy with AAV vectors has demonstrated the potential of correcting genetic disorders in a permanent manner by delivering a functional copy of a gene into the nucleus of somatic cells in affected tissues. The transferred gene, or transgene, compensates for genetic mutations underlying inherited genetic disorders. AAV vectors are particularly attractive as *in vivo* gene delivery tools, as they are mostly non-integrative and can transduce a wide variety of terminally differentiated tissues, driving long-term transgene expression,[1–5] while they are inefficient at transducing antigen-presenting cells (APCs) and have a low immunogenicity profile.[6,7] Despite this, immune responses encountered in humans undergoing gene transfer with AAV vectors have been an important obstacle to the advancement of the field (Figure 1).

The wild-type (WT) AAV is highly prevalent in the human population,[8] although exposure to this virus has not been clearly associated with any clinical pathology or disease.[9] After primary infection, WT AAV genomes can persist for years in host cells, either episomally or integrated within the host DNA, and be reactivated by a helper virus, such as adenovirus, herpesvirus, human papillomavirus, and vaccinia virus,[10–13] or a genotoxic reagent. Recent studies have linked the integration of the WT AAV genome to the development of hepatocellular carcinoma,[14,15] although to date no evidence of genotoxicity has emerged from the long-term follow-up of subjects enrolled in gene transfer studies.

Several different natural AAV serotypes have been isolated in nature,[16] which differ in the sequence of their capsid. The capsid serotype and the presence of a specific receptor on the host cells determine the tropism of each AAV serotype for a tissue (Table 1),[16,17] a property that makes AAVs versatile vectors adaptable to a broad range of therapeutic applications. AAV vectors can be manufactured according to various methods,[18] although the most common is by transfection of the HEK293 cell line with three different DNA plasmids encoding the vector genome, the *rep* and *cap* genes derived from a specific AAV serotype, and a helper plasmid.[19]

Pre-existing Immunity to WT AAV in Humans

Pre-existing Humoral Immunity

Several studies have investigated the seroprevalence of neutralizing antibodies directed against WT AAV in humans.[20–25] Seroprevalence varies geographically, with anti-AAV2 neutralizing antibodies displaying the highest prevalence, ranging from 30% to 60% of the population. Due to the broad cross-reactivity between AAV serotypes,[26] neutralizing antibodies recognizing virtually all serotypes can be found in almost all subjects.[27] This cross-reactivity reflects the amino acid sequence and structural homology across capsids of different AAV serotypes.[28]

The prevalence of total anti-AAV antibodies is close to 70% of the population for AAV1 and AAV2, 45% for AAV6 and AAV9, and 38% for AAV8.[20] Importantly, titers of anti-AAV immunoglobulin G (IgG) antibodies correlate significantly with titers of

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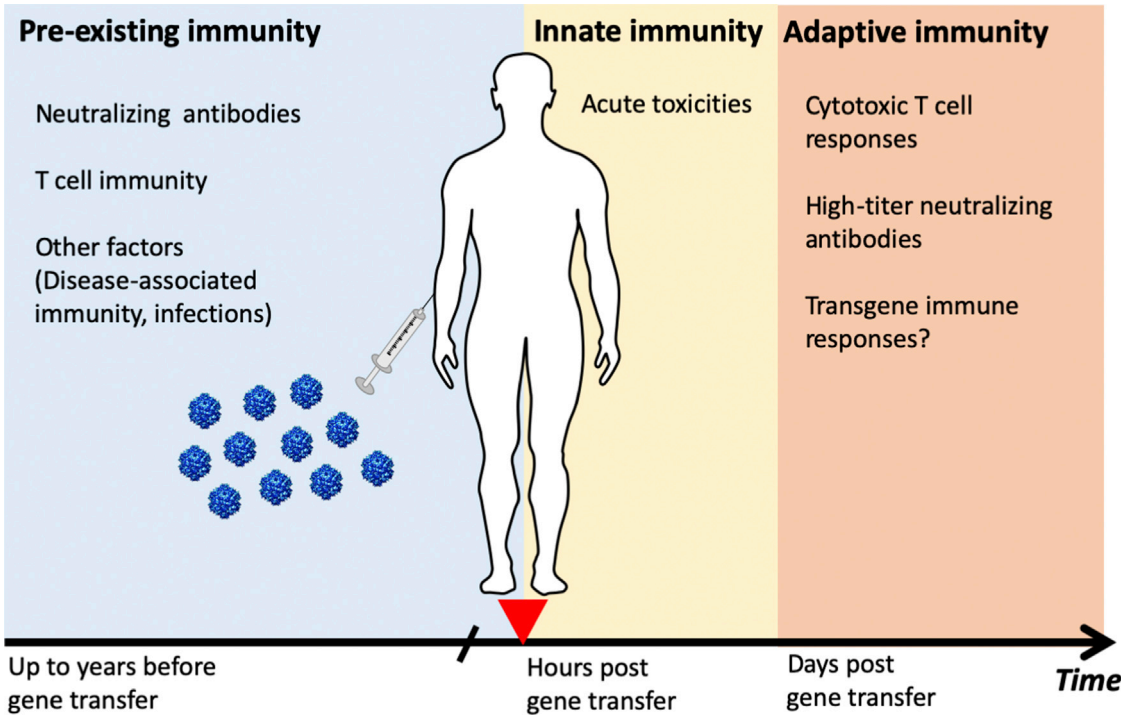

Figure 1. Host Immune Responses against AAV Vectors

Prior to vector administration, humans are exposed to wild-type AAV and therefore can develop both humoral and T cell-mediated immunity to the vector. Exposure to wild-type AAV can occur years prior to gene transfer, and together with host-specific factors can determine the overall immunological context of AAV vector delivery. Immediately after vector delivery, the vector in its components can trigger innate immune recognition. While no evidence of severe systemic inflammation has been observed in AAV trials immediately after vector delivery, some episodes of pyrexia have been documented, as well as toxicities potentially associated with complement activation. Later after vector administration, anti-capsid antibodies are produced and persist for several years after gene transfer. Capsid T cell activation has also been documented in several trials, in some cases correlating directly with loss of transgene expression. Transgene immune responses are also a potential immune-related risk in gene therapy, although to date they have been documented only in isolated trials.

anti-AAV neutralizing antibodies,[27,29] although some individuals carry non-neutralizing anti-AAV IgG.[30] Aside from the neutralizing effect on AAV vectors,[31–33] relatively little is known about the effect of antibodies on the vector tropism and immunogenicity. Systemic delivery of AAV vectors in the presence of neutralizing antibodies has been shown to result in the accumulation of vector genomes in lymphoid organs.[30,34] Conversely, in the presence of binding, non-neutralizing antibodies, the transduction efficiency of organs such as the liver appears to be enhanced.[30] An additional role of antibodies as mediators of toxicities associated with complement activation[35] in gene transfer trials is being investigated (*vide infra*), although it is known that AAV vectors interact with complement proteins.[36,37]

IgG1 appears to be the predominant immunoglobulin subclass in WT AAV seropositive individuals,[20,38] although some subjects carry high levels of IgG2 and IgG3. Titers of IgG1, IgG2, and IgM are well correlated with the level of neutralizing antibodies (Nabs), which is not the case for IgG3 and IgG4.[38] Similarly, in subjects undergoing AAV vector gene transfer, the development of high-titer IgG1 antibodies together with NAbs has been documented.[38] In contrast, levels of IgG3 have been found to be correlated with the detection of T cell reactivity to AAV vectors.[39]

Pre-existing Cellular Immunity

Since the initial findings of T cell-mediated immunity in AAV gene transfer trials,[32,40,41] the scientific community has focused on pre-existing anti-AAV cellular immunity and its potential impact on AAV-based gene transfer.[42–44]

The prevalence of T cells directed against AAV1 and AAV2 in the general population has been investigated through a variety of functional assays, which include enzyme-linked immunospot (ELISPOT)[45–47] and flow cytometry-based assays.[42,45,47] Although the prevalence can vary across studies, depending on the sensitivity of the assay, capsid-specific cellular responses are less frequent, or less detectable, than humoral responses. Several studies[40,45,47] have pointed out the lack of correlation between detection of T cells secreting interferon (IFN)γ in response to capsid antigen and the presence of anti-AAV antibodies in serum. More recently, we identified a correlation between detection of circulating capsid-specific memory $CD8^+$ T cells secreting tumor necrosis factor (TNF)-α

Table 1. Receptors and Preferential Tissue Tropism of Natural AAV Vectors

Serotype	Source	Glycan Receptor	Co-receptor/Other	Examples of Tissue Tropism
AAV1	non-human primate	N-linked sialic acid	unknown	skeletal muscle, lung, CNS, retina, pancreas
AAV2	human	HSPG	FGFR1, HGFR, LamR, CD9 tetraspanin	smooth muscle, skeletal muscle, CNS, liver, kidney
AAV3	non-human primate	HSPG	FGFR1, HGFR, LamR	hepatocarcinoma, skeletal muscle, inner ear
AAV4	non-human primate	O-linked sialic acid	unknown	CNS, retina
AAV5	human	N-linked sialic acid	PDGFR	skeletal muscle, CNS, lung, retina, liver
AAV6	human	N-linked sialic acid, HSPG	EGFR	skeletal muscle, heart, lung, bone marrow
AAV7	non-human primate	unknown	unknown	skeletal muscle, retina, CNS
AAV8	non-human primate	unknown	LamR	liver, skeletal muscle, CNS, retina, pancreas, heart
AAV9	non-human primate	N-linked galactose	LamR	liver, heart, brain, skeletal muscle, lungs, pancreas, kidney
AAV10	non-human primate	unknown	unknown	liver

and the presence of anti-AAV antibodies,[42] possibly indicating that IFNγ is not the main signature cytokine of T cell-mediated immune responses to AAV.

Capsid-specific T cells are highly cross-reactive[45] and can be detected more frequently in splenocytes compared to peripheral blood mononuclear cells (PBMCs), suggesting that AAV-specific memory T cells might fail to recirculate in peripheral blood and preferentially home to lymphoid organs.[40,45] In addition, a higher prevalence of T cell responses in PBMCs or splenocytes is observed after several rounds of *in vitro* expansion, suggesting that the frequency of AAV-specific T cells is too low to be efficiently detected *ex vivo*.[40,45] The fact that flow cytometry-based assessment of differentiation markers evidenced that most AAV-specific T cells exhibit a memory phenotype[40,42,43,47] suggests that they might arise during infancy after naturally occurring WT AAV infections, and persist throughout lifetime as a pool of memory T cells in secondary lymphoid organs. This hypothesis is consistent with the detection of reactive T cells in splenocytes from adults and, at a lower frequency, from children.[40,45]

AAV-specific memory T cells have been shown to produce IFNγ, interleukin (IL)-2, and TNF-α and to present a cytotoxic phenotype characterized by the expression of granzyme B and CD107a degranulation markers.[42,43,45,47] We recently identified two patterns of cellular responses to AAV depending on the serology of patients. By conventional and CyTOF mass cytometry,[48] we observed that exposure of human PBMCs to AAV capsid epitopes induced the activation of $CD8^+$ T cells with an effector memory phenotype, which secreted predominantly TNF-α, but also cytolytic granules containing granzyme B and CD107a.[42] Alternatively, in seronegative patients we observed transient activation of natural killer (NK) cells, but not naive $CD8^+$ T cells. These NK cells secreted both IFNγ and TNF-α, but they did not show a cytotoxic phenotype. The role of NK cells as effectors of cellular immune responses to AAV remains to be characterized.

Unlike for humoral immunity, the role of pre-existing T cell responses directed against capsid epitopes in the outcome of gene transfer is not entirely understood. The timing of detection of T cell reactivity to AAV in gene transfer trials has been inconsistent with that of a memory recall response. However, the current knowledge on immune responses in the context of an ongoing viral infection may not entirely apply to the setting of gene therapy, in which, for example, large quantities or non-replicating virions are infused directly into the bloodstream.

Induction of Immune Responses against AAV Vectors

Innate Capsid Immunogenicity

Several complex processes contribute to generating an immune response against an offending pathogen. Because AAV vectors lack any coding viral sequence, the main sources of foreign antigens brought in during gene transfer, aside from contaminant carryovers from the production and purification process, are derived from initial input of viral capsid and from the transgene product. The DNA component of AAV vectors, and possibly double-stranded RNA (dsRNA) produced by the vector itself,[49] can also act as an adjuvant, concurring in the activation of innate immunity along with other host-specific factors (Figure 2).

In recent years, innate immunity to AAV has gained increasing attention as one of the possible triggers of immune-mediated toxicities observed in clinical trials, although the overall lack of clinical evidence (e.g., detection of proinflammatory cytokines in the circulation) has been a challenge to clearly establish a direct causative relationship.

Innate immunity mounts rapidly, is non-specific, and does not result in immunological memory. Innate immune responses are initiated through the recognition of pathogen-associated molecular patterns (PAMPs), exhibited on pathogens, by PRRs (pattern recognition receptors) expressed at the surface or within immune cells. These PRRs recognize viral nucleic acids, as well as membrane glycoproteins, or even chemical messengers. Through a variety of signaling pathways, the engagement of PRRs mainly leads to the activation of NF-κB (nuclear factor κB) and IRF (IFN-regulatory factor) transcription factors, both of which play a central role in inducing the expression of pro-inflammatory cytokines or type I IFNs, respectively.[50] Type I IFNs have been described as being important for the induction of anti-capsid $CD8^+$ T cell responses.[51] In the

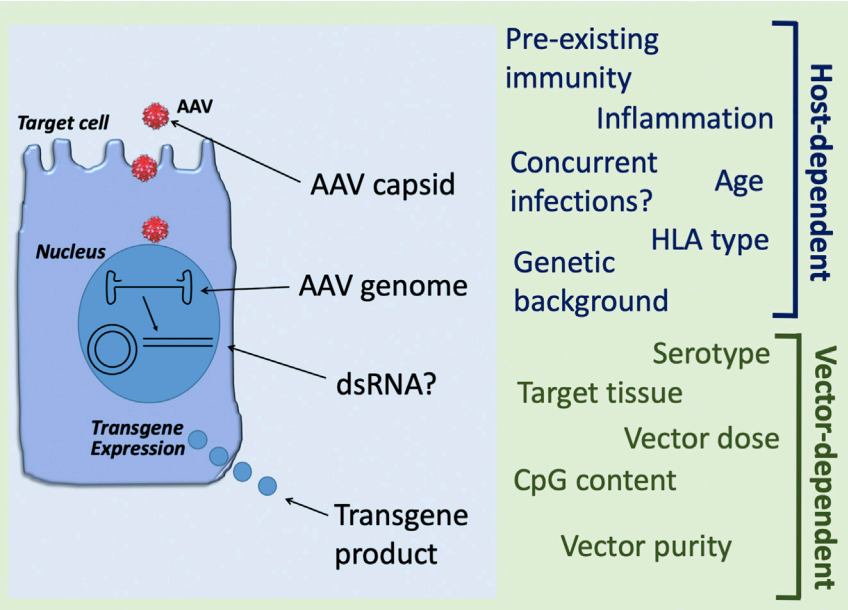

Figure 2. Factors that Influence AAV Vector Immunogenicity

The capsid, its genome, and the transgene product are the main potential immunogenic components of AAV vectors. Production of dsRNA driven by the promoter activity of ITRs can also act as a trigger for innate immunity. Additional host-dependent and vector-dependent factors can modulate the overall vector immunogenicity. These factors are mostly poorly understood, although the presence of innate immunity activators such as CpG and vector dose seem to correlate with vector-related immunotoxicities in some trials.

preclinical setting, blocking the pathways of activation of innate immune responses has been shown to prevent anti-capsid cytotoxic[51,52] and humoral responses[42] *in vivo*. In non-parenchymal liver cells, including Kupffer cells and liver sinusoidal endothelial cells (LSECs), the viral capsid has been seen to activate innate immunity mainly through binding to Toll-like receptor (TLR)2 expressed on the cell surface.[53] Moreover, the double-stranded DNA vector genome, and in particular unmethylated CpG motifs, are recognized by the endosomal TLR9 in Kupffer cells,[54] peripheral plasmacytoid dendritic cells (pDCs),[52,55] and monocyte-derived DCs.[56] TLR9 engagement has been associated with enhanced capsid antigen presentation into major histocompatibility complex (MHC) class I and subsequent capsid-specific $CD8^+$ T cell activation.[44,52,57] Also, the MyD88 (myeloid differentiating factor 88)-TLR9 pathway has been shown to mediate the induction of immune responses to liver- and muscle-targeted transgenes.[56,58]

In addition to the vector DNA, a recent study suggested a possible contribution of dsRNA to the induction of innate immunity to AAV.[49] This would explain why cellular responses are sometimes initiated weeks after vector administration in clinical trials.[5,41] According to this study, the promoter activity of the inverted terminal repeats (ITRs) flanking the transgene expression cassette could potentially drive the production of dsRNA, which in turn would stimulate the MDA5 sensor in human hepatocytes transduced with AAV, leading to the expression of type I IFN. Interestingly, blockade of MDA5 decreased the IFN response and improved transgene expression in transduced cells *in vitro*.[49]

Adaptive Immunity

Adaptive immunity occurs after innate immunity and allows the antigen-specific recognition and elimination of pathogens, followed by the establishment of immunological memory. During the establishment of an adaptive response, T and B lymphocytes become activated after recognizing an antigen presented by APCs.[59] After activation, lymphocytes expand and differentiate into effector cells and mediate the elimination of antigens through the induction of humoral or cytotoxic responses. After clearing the antigen, the adaptive immune response is followed by a contraction phase and the generation of memory T and B lymphocytes, which can become re-activated upon re-exposure to the antigen.[59]

It has been demonstrated that transduced cells and professional APCs present immunogenic epitopes derived from the capsid to cytotoxic $CD8^+$ T cells via MHC class I.[40,45,52,60] Cytotoxic T cells are then responsible for driving clearance of AAV-transduced cells, causing inflammation in the target organ, and decreasing the duration and efficacy of gene transfer.[32,41,61,62] In sync with MHC class I presentation, recognition of capsid-derived epitopes bound to MHC class II on the surface of APCs activates $CD4^+$ T helper cells, which facilitate humoral and cell-mediated immune responses.[51]

Experience from clinical trials suggests that AAV vector immunogenicity is to some extent dose-dependent,[39,63] for which low vector doses are more likely to induce a mild inflammation that can be managed and does not result in total loss of transgene expression[39] (Figure 2). This is consistent with *in vitro* studies showing dose-dependent levels of capsid antigen being presented by transduced cells onto MHC class I.[60,64] Additional factors that can drive vector immunogenicity are less known and can include pre-existing tissue inflammation,[65] the use of single- or double-stranded vector genomes,[54] and the CpG content of the vector genome[44,66] (Figure 2).

Immune Responses against the Transgene Product

Several factors contribute to shape the immunogenicity of the transgene product in AAV gene transfer. These can be divided into host-specific factors, associated with the underlying disease or the genetic background of the vector recipient, and vector-specific factors, to group factors related to gene transfer (Table 2). Similar to anti-capsid immune responses, presentation of transgene-derived

Table 2. Factors Driving Anti-transgene Immunogenicity

	Enhanced Transgene Immunogenicity	Reduced Transgene Immunogenicity	References
Host-Specific Factors			
Underlying mutation	null mutations (CRIM-negative)	missense mutations (CRIM-positive)	71,72
Disease-specific changes in target tissue	presence of inflammation, immunity against self-protein, immune system alterations	healthy	73,74,75
Previous exposure to recombinant protein	naive patients or patients with inhibitors against recombinant protein	selection of patients with no inhibitors against recombinant protein	76,77
Vector-Specific Factors			
Route of administration	intramuscular	systemic, immunoprovileged organ	72,78
Promoter	strong, constitutive, muscle specific	liver-specific	79,80,81
Vector genome	self-complementary, CpG rich, dsRNA	single stranded, CpG low, disruption of ITR promoter activity	49,54,58,66
Transgene	intracellular, highly glycosylated, large	secretable, native glycosylation pattern, small	81,82–84
Vector dose	low hepatocellular expression	high hepatocellular expression	85

epitopes to $CD8^+$ and $CD4^+$ T lymphocytes can induce cytotoxic and humoral responses that have a negative impact on transgene stability.[56,58,67,68] One key factor determining the level of anti-transgene immune responses is the target organ for gene transfer, which is determined by the combination of the AAV capsid, the vector delivery route, and the tissue specificity of the promoter driving gene expression. In particular, systemic and intramuscular vector administration, with either ubiquitous or muscle-specific promoters, have been shown to be more immunogenic than gene transfer to immune privileged organs, as well as systemic administration with liver-specific promoters[69,70] (Table 2).

In the context of AAV trials, anti-transgene immune responses have been documented in only a few instances, mostly in the context of intramuscular delivery of AAV vectors. The first evidence of transgene-specific cytolytic T cell activation came from a phase I/II trial of intramuscular gene transfer of a microdystrophin transgene.[73] In this trial, lack of transgene expression was associated with the development of a $CD8^+$ T cell responses directed against epitopes mapping within the transgene amino acid sequence. Similarly, in a trial for α-1 antitrypsin (AAT) deficiency, following intramuscular delivery of an AAV vector, cytolytic T cells were detected in association with decreased transgene expression in one of the participants,[86] although the trial showed overall long-term expression of the transgene in most participants.[4] More recently, in the context of systemic delivery of an AAV vector for the treatment of X-linked myotubular myopathy, anti-transgene antibodies were detected,[87] although with no direct correlation with clinical endpoints (*vide infra*). Finally, transgene-reactive T cells were also detected in a phase I/II trial of intracranial delivery of an AAV5 vector to the brain of children affected by mucopolysaccharidosis type IIIB.[88]

The significance of these early findings and their impact on endpoints of safety and efficacy are not entirely clear. What appears to be consistent is that targeting tissues that are not immune privileged, such as the muscle,[89] with gene transfer may pose additional challenges related to transgene immunogenicity. To this end, concomitant expression of a transgene in a protolerogenic tissue such as the liver, at the same time as muscle, may help transgene engraftment.[69,79]

The Tolerogenic Potential of Liver-Directed Gene Transfer

In the context of liver-directed gene transfer, transgene immunogenicity appears to be less of a potential concern compared to other tissues. Starting with the initial observation that mice expressing human factor IX (F.IX) in liver were immunologically tolerant to the transgene product,[80] several studies with AAV vectors in small and large animal models of genetic diseases show that expression of an antigen in hepatocytes can promote robust antigen-specific immune tolerance (Figure 3).[80,90,91]

Although several laboratories have investigated the mechanisms driving liver tolerance, results are not fully overlapping and depend on the experimental setting and model antigen used. The tolerogenic effect of liver gene transfer is likely to be mediated by the different cell types that can act as APCs in the liver. Reports indicate that liver-resident macrophages (Kupffer cells) and DCs have a less mature phenotype compared to professional APCs found in the periphery.[93,94] This property seems to make them poor T cell activators[95] as antigens are presented in the absence of sufficient co-stimulator ligands. Furthermore, it has been shown that Kupffer cells can secrete IL-10, an anti-inflammatory cytokine, upon TLR stimulation.[96–98] Another particularity is that LSECs are able to act as professional APCs presenting antigens through MHC class II, which seems to play a role in the induction of regulatory T cells (Tregs).[99,100] Several studies have demonstrated the essential role of Tregs in the induction of liver-mediated tolerance to liver-targeted transgenes (Figure 3).[69,90,91,101] In these studies, disruption of Treg homeostasis around the time of vector administration led to an immune response against the transgene. Conversely, administration of rapamycin, a drug known to favor Treg expansion,[102] enhanced efficiency of induction of tolerance in the context of established immunity.[103,104]

Tregs are not the only player involved in liver tolerance. $CD4^+$ T cell anergy,[105] apoptosis of reactive T cells,[80,106] degradation of T cells in hepatocytes,[107] induction of $CD8^+$ Tregs,[108] and the acquisition of an exhausted phenotype by cytotoxic $CD8^+$ T cells[69,85,109,110] have also been described in the context of hepatocellular antigen presentation

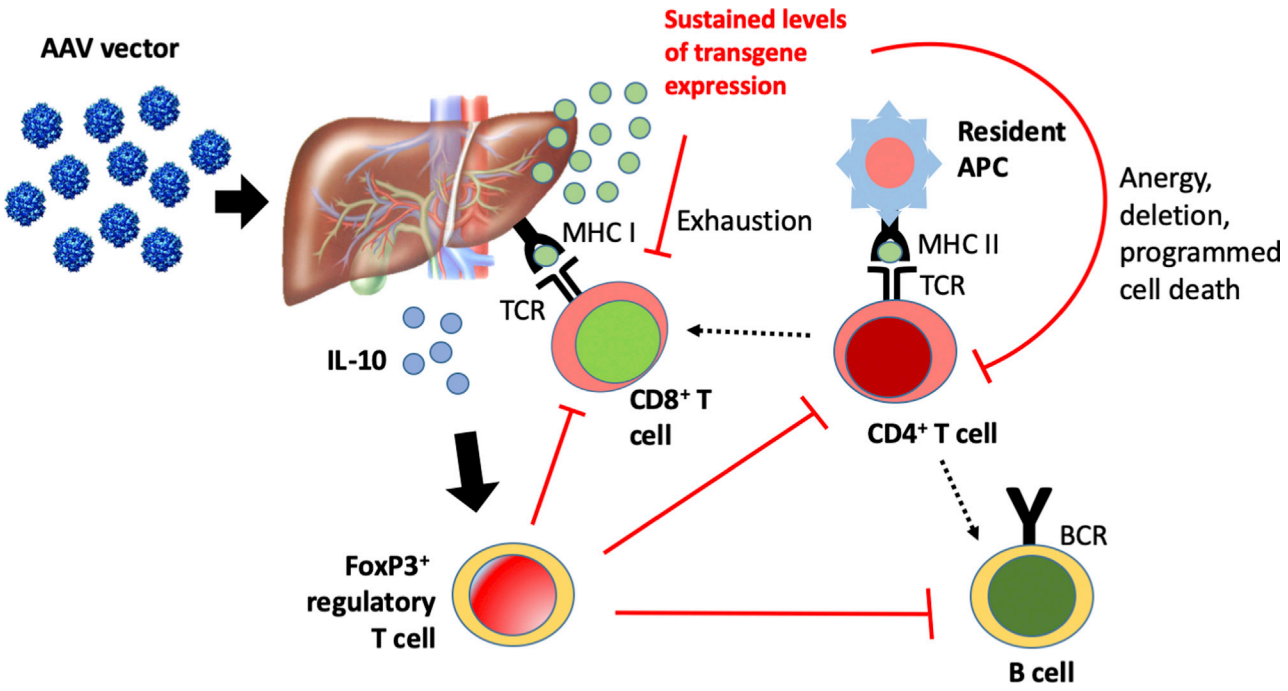

Figure 3. Liver Gene Transfer Can Drive Transgene Immune Tolerance

Tolerance to a variety of transgenes expressed in the liver is mediated by a variety of mechanisms. Tregs are a common denominator of liver tolerance, as they mediate the suppression of both humoral and T cell-mediated transgene immune responses. Additional mechanisms include anergy, exhaustion, and deletion of reactive T cells. Key to tolerance induction appears to be a robust transgene expression in hepatocytes. Adapted from Sherman et al.[92]

(Figure 3). Induction of liver tolerance has been shown to be dose-dependent,[111] as high levels of antigen presentation by hepatocytes through MHC class I have been associated with a more efficient induction of $CD8^+$ T cell exhaustion and apoptosis,[85,112] although in the context of AAV gene transfer this has not been fully demonstrated.

While preclinical data on liver tolerance are convincing, with compelling data on immunity eradication in small and large animal models of hemophilia[76,77,111] and other diseases,[79,113] the open question is whether this concept will translate to humans carrying pre-existing immunity against a given therapeutic transgene.

Immune Responses to AAV Vectors in Clinical Trials

Recombinant AAV vectors are relatively simple from an immunogenicity point of view, as they do not encode viral proteins and they comprise a protein capsid and a DNA genome, which can be single or double stranded. Pre-existing immunity originating from the exposure to WT AAV, however, can generate both humoral and cell-mediated immunity to the virus, which can cross-react with AAV vectors. While pre-existing humoral immunity represents one of the most efficient barriers to prevent successful gene transfer through systemic administration of AAV vectors (Figure 4),[114] the impact of pre-existing T cell immunity to AAV is not fully understood.

Impact and Relevance of Anti-AAV Antibodies

Whereas cytotoxic responses can mostly be controlled by transient immunosuppression,[5,63] the elevated NAb titers induced by AAV administration prevent vector readministration, particularly upon systemic delivery (Figure 4). This is a potential cause of concern when treating infants,[115] since transgene expression following AAV gene transfer is expected to decrease due to tissue growth and dilution of the vector genome.[116,117] Furthermore, pre-existing immunity has been shown to prevent cell transduction by AAV vectors,[31,32,118] for which the presence of NAbs above a certain threshold is currently an exclusion criterion for the enrollment of patients in AAV-mediated gene therapy clinical studies. Thus,humoral responses against the AAV capsid are still a major hindrance to the clinical application of this *in vivo* approach, although solutions to the problem are being tested in preclinical models.[114,119]

Ocular Gene Transfer

The eye is a highly compartmentalized organ whose local environment is anatomically isolated from the peripheral immunity by the blood-ocular vasculature, and by the absence of lymphatic vessels.[120,121] Therefore, in the context of gene transfer, the eye has been long thought to be at lower risk for activating innate and adaptive immune responses upon vector administration. Moreover, a phenomenon known as anterior chamber-associated immune deviation (ACAID) has been described in the eye upon recognition of immunogenic antigens, which involves the induction of Tregs,

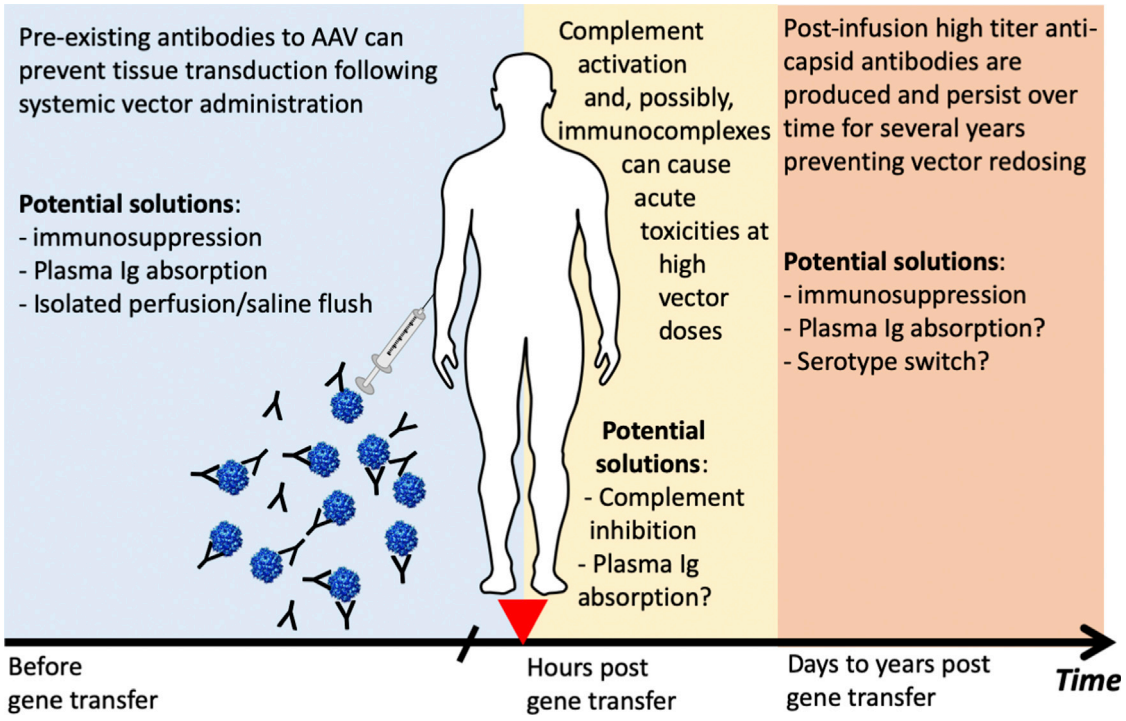

Figure 4. Humoral Immune Responses to AAV

Pre-existing immunity to AAV can block target tissue transduction when the vector is administered systemically directly into the bloodstream. While eradication of humoral immunity with immunosuppressive drugs can be challenging, as pharmacological targeting of B cells has inherent risks, pre-clinical evaluation of physical removal of antibodies with plasmapheresis has shown promising results. Isolation of target organs at the time of vector administration has also been explored. Recent data linked the acute toxicities observed following systemic administration of AAV vectors with complement activations. These toxicities may be mediated by anti-AAV antibodies and can be modulated by drugs targeting the complement activation pathways. Finally, after gene transfer, antibodies to AAV are induced and persist for the long term. Several potential approaches to vector readministration have been explored preclinically, with variable degrees of success.

anti-inflammatory M2 macrophages, and the generation of an anti-inflammatory cytokine environment that promotes immunological tolerance.[122,123] This pro-tolerogenic environment is a protective adaptation aimed at preventing inflammatory responses that could affect vision. An immune deviation phenomenon similar to ACAID has been reported in the context of AAV vector administration to the subretinal space.[124]

The well-characterized immune privilege, the fact that circulating antibodies directed against the AAV capsid are not normally found in the eye,[125] and the relatively low vector doses required to achieve therapeutic efficacy have driven the early successes of ocular gene transfer in the clinic.[126,127] Based on these promising results, and banking on more than two decades of research in the field of ocular gene transfer, in recent years the field has experienced a dramatic expansion, with several gene transfer trials planned and ongoing, mostly based on the AAV vector platform.[128]

To date, AAV vector administration has been mainly performed intravitreally or subretinally (Figure 5). Based on preclinical animal models and clinical trials, immunogenicity outcomes are greatly influenced by the route of vector administration to the eye, with subretinal vector delivery being less immunogenic than intravitreal administration.[129] However, depending on the total vector dose administered, inflammatory responses can be detected regardless of the route of vector administration (*vide infra*).

Gene Transfer to the Subretinal Space

Most progress in the subretinal delivery of AAV vectors has been made in the treatment of Leber's congenital amaurosis type 2 (LCA2) caused by mutations in the retinal pigment epithelium-specific 65-kDa protein (RPE65) gene. So far, all reported studies have been based on AAV2 vectors,[126,127,130] except for one study in which an AAV4 was administered.[131] Herein, we discuss mainly the results from the LCA2 trials, which share similarities in the design of the RPE65 expression cassette and route of vector administration.

In a study sponsored by the University College London (ClinicalTrials.gov: NCT00643747), three young adult participants were injected subretinally with an AAV2 vector carrying the *RPE65* transgene under the control of the endogenous *RPE65* promoter, at the dose of 1×10^{11} vector genomes (vg)[126] (Table 3). Patients

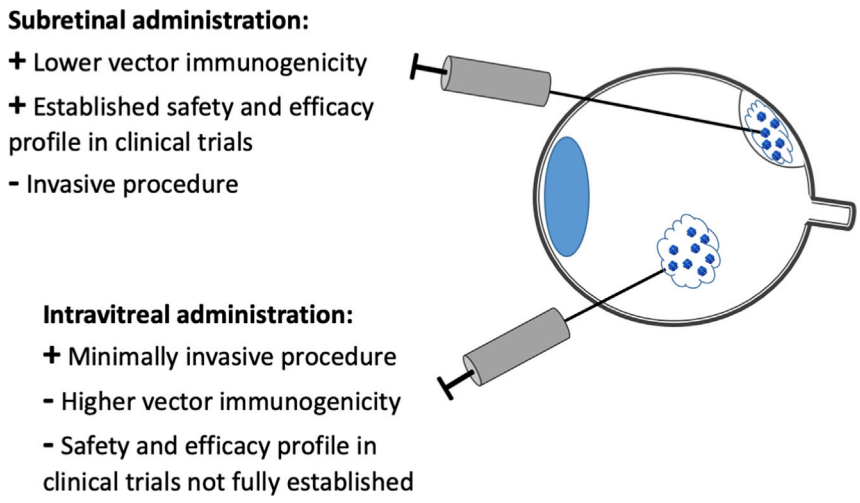

Figure 5. AAV Vector Administration to the Eye
Two main routes of vector administration to the eye have been explored. Subretinal administration has been tested in several trials and has a demonstrated safety and efficacy profile in humans. While the approach appears to be feasible, safe, and associated with a low vector immunogenicity profile, it required a surgical procedure that is relatively invasive. Conversely, intravitreal vector administration requires a simple procedure for vector delivery. This route of administration seems to result in higher vector immunogenicity, resulting in inflammation after vector delivery. The safety and efficacy profile of intravitreal delivery of AAV vectors is being evaluated in several trials.

received tapering immunosuppressive treatment with oral prednisolone for 5 weeks, starting 1 week prior to vector administration. No capsid- or transgene-specific immune responses were detected.[126] After this first report, nine more participants were included in the study and treated at 1×10^{11} or 1×10^{12} vg, with data of 3 years follow-up published in 2015[132] (Table 3). Overall, transient improvements in retinal sensitivity were observed in 6 participants at 6–12 months post-injection, but they appeared to decline over time. Immune-related events occurred in five out of eight participants from the high-dose cohort. Two of them developed detectable anti-AAV2 NAbs, and one was also positive for IFNγ responses to the capsid measured by ELISPOT at 4 weeks post-injection. In the participant with a positive ELISPOT, inflammation was associated with a worsening in visual function in the injected eye. No immune responses to the transgene were detected.

In a study conducted by the University of Pennsylvania (ClinicalTrials.gov: NCT00481546), three participants initially received a single subretinal injection of an AAV2 at 5.96×10^{10} vg, in with the expression of the RPE65 transgene was regulated by the constitutive chicken beta actin (CB) promoter containing an optimized cytomegalovirus (CMV) enhancer (AAV2-CB^{SB}-hRPE65) (Table 3).[130,141] In this study no systemic immunosuppression was administered, and a mild increase in humoral and cellular responses to the AAV2 capsid was detected in one participant. Twelve more subjects were treated with this vector in a dose-escalation study (Table 3).[133] Most participants presented anti-AAV2 antibodies prior to vector administration, and seven patients had pre-existing AAV2-specific T cells at baseline. Nevertheless, only 4 out of 15 subjects experienced a significant increase of circulating anti-AAV2 antibody titers, and one had a positive IFNγ ELISPOT on day 90, possibly highlighting the limited potential of antigens delivered to the eye to stimulate peripheral memory responses. Administration of two vector boluses in the same eye did not appear to result in enhanced immunogenicity. Despite the promising results in visual sensitivity, long-term evaluation performed after 5–6 years of treatment showed that visual function tended to peak between 1 and 3 years, and declined afterward, in some subjects returning to baseline.[142]

In the work initially sponsored by the Children's Hospital of Philadelphia and later by Spark Therapeutics (ClinicalTrials.gov: NCT00516477), the first report from 2008 involved three young adult LCA2 patients treated by subretinal injection with an AAV2-CB-hRPE65v2 vector containing an optimized Kozac sequence, at 1.5×10^{10} vg in one eye, with concomitant local and systemic corticosteroid immunosuppressive treatment (Table 3).[127] No significant immune responses were detected against capsid or transgene, except for a transient increase in anti-AAV2 antibodies in one participant. Twelve additional participants were included in higher dose cohorts of 4.8×10^{10} and 1.5×10^{11} vg.[134,135,143] Two out of 3 participants from the high-dose cohort had mild and transient activation of capsid-specific T cell responses detectable in peripheral blood, and 6 out of 12 had a transient increase in anti-AAV2 NAbs. In a follow-up study, 11 previously treated patients received a second vector injection in the contralateral eye (ClinicalTrials.gov: NCT01208389). All subjects were injected with the highest vector dose in the second eye (1.5×10^{11} vg), between 1.7 and 4.58 years after the first injection (Table 3). Results showed no significant induction of cellular or humoral memory responses against the vector or transgene induced after vector readministration, further confirming the low immunogenicity profile of subretinal delivery of AAV vectors.[2,136]

A phase III study was later initiated (ClinicalTrials.gov: NCT00999609), and 29 subjects were treated subretinally in both eyes at 1.5×10^{11} vg (Table 3).[137,138] All participants received oral treatment with prednisolone starting prior to the first injection and continuing until the time of the second injection, which took place 6–18 days after the first procedure. No signs of significant immune responses were documented, except for a mild eye inflammation observed in two participants. The treatment was recently approved by the US Food and Drug Administration (FDA) and

Table 3. Overview of Clinical Studies Based on Sub-retinal Delivery for the Treatment of LCA2

Sponsor	Phase	Study ID: ClinicalTrials.gov	Product	Dose (vg)	Preventive IS	Immune Responses	References
University College London	I/II	NCT00643747	AAV2-RPE65-RPE65	1×10^{11} (n = 4) 1×10^{12} (n = 8)	yes	NAbs in one participant; inflammatory responses in five participants	126,132
University of Pennsylvania	I	NCT00481546	AAV2-CB^{SB}-RPE65	5.96×10^{10} (n = 3) 8.94×10^{10} (n = 3) 11.92×10^{10} (n = 3) 17.88×10^{10} (n = 5) $7.95x10^{10}$ (n = 1)	no	anti-capsid pre-existing humoral and cellular immunity; humoral response in four participants; cellular response in one participant	130,133
Spark Therapeutics	I	NCT00516477	AAV2-CB-hRPE65v2 (voretigene neparvovec)	1.5×10^{10} (n = 3) 4.8×10^{10} (n = 6) 1.5×10^{11} (n = 3)	yes	induction of anti-AAV2 NAbs in six participants; cellular responses in two participants	127,134,135
Spark Therapeutics	I	NCT01208389	AAV2-CB-hRPE65v2 (voretigene neparvovec)	1.5×10^{11} in the contralateral eye (n = 11)	yes	no significant immune responses	2,136
Spark Therapeutics	III	NCT00999609	AAV2-CB-hRPE65v2 (voretigene neparvovec)	1.5×10^{11} in both eyes (n = 29)	yes	mild ocular inflammation in two participants	137,138
Applied Genetic Technologies	I/II	NCT00749957	AAV2-CB^{SB}-RPE65	1.8×10^{11} (n = 6) 6×10^{11} (n = 6)	no	ocular inflammation in two participants	139,140
Nantes University Hospital	I/II	NCT01496040	AAV4-RPE65-RPE65	1.22×10^{10} (n = 1) 1.82×10^{10} (n = 2) 3.23×10^{10} (n = 1) 4.27×10^{10} (n = 2) $4.7–4.8 \times 10^{10}$ (n = 3)	yes	anti-AAV4 IgG in three patients; ocular inflammation in three patients; cellular immune responses against capsid and transgene in one participant	131

IS, immunosuppression.

European Medicines Agency (EMA). The drug product is marketed as Luxturna and manufactured by Spark Therapeutics.[144] A long term-follow up study is currently open (ClinicalTrials.gov: NCT03597399).

Additional trials have been conducted for the treatment of LCA2 based on AAV2 (ClinicalTrials.gov: NCT00749957 and NCT00821340),[139,140] AAV4 (ClinicalTrials.gov: NCT01496040),[131] or AAV5 (ClinicalTrials.gov: NCT02946879 and NCT02781480) vectors encoding RPE65.[128,145] Among the studies with recently published results, one study sponsored by the Nantes University Hospital was based on AAV4,[131] which presents specific tropism for RPE cells (Table 3). The transgene was expressed under the endogenous RPE65 promoter, and the vector was administered in multiple injections in one eye in the absence of immunosuppression. Anti-AAV4 antibodies were observed in three out of nine patients, three of them also experienced transient inflammation, and one patient showed detectable IFNy responses against both capsid and transgene.[131]

Several additional clinical studies of subretinal delivery of AAV vectors are ongoing for indications that include choroideremia,[146,147] X-linked retinitis pigmentosa,[148] neovascular ("wet") age-related macular degeneration (wAMD),[149–151] and others.[128] Overall, these studies evidence that immune responses against AAV occur in a fraction of patients despite the immune privilege of the eye and the use of immunosuppression. Anti-transgene immune responses are rare, but they were also reported in one study.[131]

Currently, administration of corticosteroids is broadly used as a measure to limit both the inflammation derived from the administration procedure and to modulate vector immunogenicity. Despite this, instances of inflammation have been observed across trials, and particularly as higher doses of vector were administered. Potential solutions have been proposed to decrease vector immunogenicity,[152] and it is likely that more effective solutions than corticosteroid administration will be needed to tackle gene therapy strategies requiring high vector doses, such as those relying on dual AAV vectors.[153] Similar considerations are also likely to apply to the field of gene editing, as they often rely on AAV vectors as vehicles for donor templates[154–157] and because of the potential issues related to the immunogenicity of Cas9.[158,159]

Intravitreal AAV administration

Intravitreal vector administration has been preclinically explored for a variety of indications, based on the ease of vector administration through this route (Figure 5) and on the need, for some indications, to target specific cell types.[160,161] The potential for some

AAV vector serotypes to reach the outer layers of the retina[162] has further heightened the interest on this route of vector administration.

Intravitreal vector administration has been explored in in the context of few clinical trials. Wan et al.[161] evaluated the safety and efficacy of an AAV2 vector encoding for mitochondrial reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase 4 gene (ND4) administered to the vitreous in nine Leber's hereditary optic neuropathy (LHON) patients (ClinicalTrials.gov: NCT01267422). A 9-week course of prednisolone treatment was given around vector administration and subjects with high-titer, pre-existing NAbs were excluded. No significant humoral or cellular immune responses against the vector were documented in this study, which also showed no evidence of therapeutic efficacy. In another study, unilateral intravitreal injection of a self-complementary AAV2-ND4 vector was performed in 14 LHON patients, in the absence of immunosuppression (ClinicalTrials.gov: NCT02161380).[160,163] Neutralizing antibodies to AAV2 were detected in all participants prior to injection, and increased afterward at variable titers, and in two subjects development of high antibody titers were associated with anterior uveitis. Finally, in a phase I/II study sponsored by GenSight Biologics (ClinicalTrials.gov: NCT02064569), an AAV2-*ND4* vector was administered by a single intravitreal injection at increasing doses in the absence of corticosteroids.[164,165] Most participants experienced inflammation in the anterior chamber and vitreous, which has not been directly associated with the vector dose, and two participants were given oral corticosteroids to modulate the response. Levels of anti-capsid antibodies increased after injection in most patients enrolled in the trial.[164,165] Follow-up of LHON patients enrolled in a phase III trial is currently ongoing. In this study a bilateral injection of the vector at 9×10^{10} vg/eye was performed (ClinicalTrials.gov: NCT03293524).

Additional studies of intravitreal gene transfer of AAV vectors were conducted in the context of wAMD. Heier et al.[166] used an AAV2 to deliver the soluble vascular endothelial growth factor (VEGF) receptor sFlt-1 (AAV2-sFLT01) at doses between 2×10^8 and 2×10^{10} vg in 19 subjects (ClinicalTrials.gov: NCT01024998). Two patients in the high-dose cohort 4 experienced pyrexia and intraocular inflammation upon vector administration, which was treated with topical steroids. Transgene expression was detected in 5 out of 10 subjects who had low or no pre-existing NAbs, which peaked on week 26 and declined during the 52-week follow-up period. The lack of transgene expression in the remaining patients correlated with the presence of high antibody titers against AAV2 prior to vector administration.[166] Similarly, in an ongoing trial for wAMD sponsored by Adverum Biotechnologies, vector doses up to 6×10^{11} vg were tested (ClinicalTrials.gov: NCT03748784) along with a tapering course of corticosteroids. Instances of ocular inflammation were observed at the highest vector dose tested, prompting the extension of the cohort receiving a vector dose of 2×10^{11} vg.

Similar to the subretinal delivery of AAV vectors, inflammation caused by intravitreal delivery of AAV vectors appears to be dose-dependent. Thus, similar considerations apply to the need to better understand the determinants of vector immunogenicity in this context, to allow the development of strategies to overcome inflammation. At a minimum, manufacturing technologies that allow the elimination of or greatly reduce the content of non-infectious vectors particles (i.e., empty capsids) would likely be beneficial.

Liver Gene Transfer

The liver has been a long-suited target for gene transfer, as research efforts have been directed to targeting the liver for the correction of a variety of genetic and metabolic diseases[167–171] and for other applications in which, for example, hepatocytes are turned into biofactories to supply protein therapeutics directly into the bloodstream.[5,81,172–174] Several important features make the liver an ideal organ for gene therapy, including (1) the fact that hepatocytes are central to several metabolic functions and secrete a variety of proteins into the circulation; (2) its high degree of vascularization, which allows for easy transduction with any gene therapy vector delivered thorough the bloodstream; and (3) its unique immune protolerogenic environment (*vide supra*).

The first evidence of immune responses in the context of liver gene transfer was reported in a trial conducted by the Children's Hospital of Philadelphia and Avigen, in which seven subjects with severe hemophilia B received a single-stranded AAV2 vector carrying the F.IX transgene under the control of a liver-specific promoter, through the hepatic artery (Table 4).[32] Patients were divided into three dose cohorts of 8×10^{10}, 4×10^{11}, and 2×10^{12} vg/kg, respectively. Transient elevation of F.IX activity at levels around 10% of normal was detected only in the first patient treated at the highest dose. Differently from animals models, in which long-term expression is observed after liver gene transfer with AAV vectors,[41,175] 4 weeks after gene transfer, F.IX expression started to decline and eventually returned to pre-treatment level. A self-limited transient and asymptomatic rise in liver transaminases was also detected in the same time frame, suggesting the induction of cytotoxic immunity against the AAV2 capsid.[32] Similar series of events were observed in a participant enrolled in the mid-dose cohort (subject G). An IFNγ ELISPOT assay and flow cytometry-based detection of AAV-specific $CD8^+$ T cells confirmed a T cell response to the AAV2 capsid, which had a kinetics of appearance that closely mirrored the rise in serum transaminases and decrease in transgene expression.[32,40] An additional important finding from this trial was that a second subject dosed with 2×10^{12} vg/kg, a dose expected to result in detectable transgene expression, but carrying pre-existing anti-AAV NAbs at a titer of just 1:17, had no evidence of liver expression.[32]

Based on the learning from this first trial, and on preclinical data showing the potential superiority of the AAV8 serotype in transducing the hepatocytes,[182] a second clinical study was initiated a few years later (ClinicalTrials.gov: NCT00979238). The vector genome carried a self-complementary genome, a strategy

Table 4. Overview of Hemophilia B Clinical Studies Based on Liver-Directed Gene Transfer

Sponsor	Phase	Study ID: ClinicalTrials.gov	Product	Dose (vg/kg)	Immune Responses	References
Avigen	I/II	NCT00076557	ssAAV2-LP1-FIX	8×10^{10} (n = 2) 4×10^{11} (n = 3) 2×10^{12} (n = 2)	pre-existing NAbs; dose-dependent cellular responses to the capsid and elevation of liver transaminases	32
St. Jude Children's Research Hospital	I	NCT00979238	scAAV8-LP1-FIXco	2×10^{11} (n = 2) 6×10^{11} (n = 2) 2×10^{12} (n = 6)	anti-capsid cellular responses; elevation of liver transaminases in four patients resolved with prednisolone	5,63
Baxalta/ Shire	I/II	NCT01687608	scAAV8-TTR-FIXR338Lopt (AskBio009, BAX 335)	2×10^{11} (n = 2) 1×10^{12} (n = 4) 3×10^{12} (n = 2)	dose-dependent T cell responses and elevation of liver transaminases in two patients resolved with prednisolone	176,177
UniQure	I/II	NCT02396342	scAAV5-hAAT-FIX (AMT-060)	5×10^{12} (n = 5) 2×10^{13} (n = 5)	elevation of liver transaminases in three patients resolved with prednisolone	178
UniQure	IIb	NCT03489291	scAAV5-hAAT-FIX^{Padua} (AMT-061, etranacogene dezaparvovec)	2×10^{13} (n = 3)	pre-existing NAbs; no significant inflammatory responses	179
Pfizer	I/II	NCT02484092	SPK-9001	5×10^{11} (n = 15)	elevation of liver transaminases in two patients resolved with prednisolone	62,180
Dimension Therapeu-tics	I/II	NCT02618915	ssAAVrh10-FIX (DTX101)	1.6×10^{12} (n = 3) 5×10^{12} (n = 3)	elevation of liver transaminases treated with prednisolone	181

to enhance hepatocyte transduction.[183] In addition, the F.IX sequence was codon optimized to further improve transgene expression. Six seronegative participants with severe hemophilia B were infused an AAV8 vector encoding the self-complementary, codon-optimized, liver-targeted F.IX transgene in a peripheral vein.[63] In the low- and intermediate-dose cohorts, F.IX activity remained between 1% and 4% of normal, respectively, in the absence of immune-associated events. In the highest dose cohort (2×10^{12} vg/kg), however, F.IX activity reached maximum levels of 8%–10% of normal, and patients again experienced an elevation of liver enzymes at 8 weeks, which coincided with partial decline in transgene expression and a rise in circulating capsid-specific T cells detected by an IFNγ ELISPOT assay. The elevation of liver enzymes was resolved with tapered treatment with high-dose steroids, after which transgene expression remained stable.[63] Four more patients were enrolled in the high-dose cohort, two of which were transiently treated with prednisolone after experiencing a decline in F.IX expression,[5] after which transgene expression remained stable with a documented duration of up to 10 years (U.M. Reiss, 2019, National Hemophilia Foundation, conference).

Although effective at the currently used vector doses, ongoing studies will address whether this corticosteroid regimen will be effective at higher vector doses and with different AAV serotypes. Of note, evidence of T cell activation in response to AAV capsid epitopes was documented in PBMCs isolated from participants from all dose cohorts, although liver toxicity was documented only in the high-dose cohort. High levels of anti-AAV8 antibodies could be detected in all participants following vector administration.

A similar set of clinical results was also recently published in the context of another hemophilia B clinical trial in which a bio-engineered AAV capsid was administered to seronegative hemophilia B subjects at a dose of 5×10^{11} vg/kg. At this dose, 2 out of 10 participants required the administration of corticosteroids to manage vector immunogenicity, resulting in long-term transgene expression in all subjects enrolled.[62]

Corticosteroids given reactively have been broadly used across trials to modulate capsid reactivity. However, this measure did not succeed in all studies in rescuing transgene expression. In a phase I/II study initially sponsored by Baxalta/Shire (ClinicalTrials.gov: NCT01687608), a self-complementary AAV8 vector[176] was given to eight hemophilia B subjects at doses ranging from 2×10^{11} to 3×10^{12} vg/kg. Transgene expression was transient in most subjects in this study, with the exception of one subject who did not experience an instance of liver enzyme elevation and expressed the F.IX transgene over the long term. Exome sequencing analysis in this subject revealed the presence of a polymorphism potentially affecting the immune system, which could explain the lack of immune responses detected after gene transfer.[184] Similarly, a phase I/II study sponsored by Dimension Therapeutics was halted due to transaminase elevation and loss of transgene expression not controlled by the administration of high-dose corticosteroids.[181] Additional emerging data from other liver trials (ClinicalTrials.gov: NCT02991144, NCT03636438, NCT03517085, NCT03970278, and NCT03223194), and also trials in which high doses of AAV vectors are given systemically for the treatment of diseases not affecting the liver (ClinicalTrials.gov: NCT03368742 and NCT03362502),[185,186] show instances of liver toxicities that are likely to be related to vector immunogenicity.

A somewhat different outcome was documented in the context of trials in which AAV5 vectors were used to target the liver. This capsid serotype was first used in subjects affected by acute intermittent porphyria.[171] In this study, vector doses up to 1.8×10^{13} vg/kg were administered, with no evidence of transaminase elevation and no detection of capsid-specific T cells, although also no clear evidence of therapeutic efficacy. Two additional studies were published, in which AAV5 vectors produced in a baculovirus system were infused at doses up to 5×10^{12} vg/kg[178] and 6×10^{13} vg/kg[187] for the treatment of hemophilia B and A, respectively. In these two studies, elevated transaminase levels were detected and treated with corticosteroids, although their direct impact on transgene expression was not clearly established in the time frame of the initial observation period. Longer follow-up of participants in the hemophilia A trial showed a slow decline of expression during a few years after gene transfer, for which the exact etiology remains unknown.

AAV Vectors and Liver Toxicities: Lessons Learned and Open Questions

Enormous progress has been made from the first liver gene transfer trial for hemophilia B. A deeper understanding of some of the factors driving immune responses has provided tools to modulate AAV vector immunogenicity. Many lessons learned, with some important open questions, include the following:

It is now well accepted that AAV capsid can trigger dose-dependent immune toxicities that can limit the duration of transgene expression in hepatocytes. Some exceptions remain, such as that of AAV5, although the fact that this serotype is known to be less efficient than others in transducing hepatocytes may explain the higher threshold for T cell activation.

Vector design has an equally important role as the vector dose as a determinant of immunogenicity. Emerging data from clinical trials and preclinical models[44,58] indicate, for example, that the content of CpG in the vector genome greatly increases vector immunogenicity.

The liver is the first-line target organ when AAVs are administered systemically. Thus, immune-mediated liver toxicities can be encountered any time an AAV vector is given in a high dose and systemically.[186]

Long-term duration of liver-mediated expression for up to 10 years has been demonstrated in the context of hemophilia B trials. For other liver gene transfer applications, such as hemophilia A, data are still being gathered,[188] and the factors driving vector persistence are not entirely understood.

Different manufacturing methods[18] result in vector preparations with impurities at levels that can vary in nature and quantity. Some of these impurities can play a role as immunogenicity determinants, including excess of non-infectious capsids, DNA contaminants,[189] or potential protein carryover.[190]

Finally, the role of pre-existing immunity to AAV needs to be better understood in its potential role as a determinant of vector immunogenicity.

Muscle Gene Transfer

Muscle is an important target tissue for gene transfer to treat a variety of neuromuscular,[191] cardiac,[192] and metabolic diseases.[193] Moreover, even for diseases not affecting the muscle, targeting the muscle with AAV vectors has been shown to yield promising results.[194–196] In this section we focus on muscle gene transfer. Diseases mostly affecting motor neurons are discussed separately.

From an immunological perspective, the muscle has unique challenges (reviewed in detail by Boisgerault and Mingozzi[89]). For example, the inflammation associated with dystrophies can result in enhanced transgene immunogenicity.[73] Conversely, intramuscular gene transfer has been shown to induce the recruitment of $FoxP3^+$ Tregs, which may play a role in contributing to the stability of transgene expression.[56,197] Additional challenges related to targeting the muscle include the limited distribution of AAV vectors delivered intramuscularly[198,199] and the need for high vector doses to achieve widespread transduction of skeletal muscle across the entire body.

Intramuscular Delivery of AAV Vectors

The first trial in which an AAV vector was injected intramuscularly in humans was a study in hemophilia B (Table 5)[118,200] in which eight adults received an AAV2 vector encoding for F.IX intramuscularly at doses up to 1.8×10^{12} vg/kg. Administration of the vector was well tolerated with no documented immune-related toxicities (although no specific assays were used to measure T cell responses to the AAV capsid) and no evidence of vector neutralization in participants with pre-existing NAbs to AAV2. Transgene expression was persistent for the long term,[3] although it failed to reach therapeutic levels,[118,200] highlighting the challenge of administering high and therapeutically relevant doses of AAV vector intramuscularly while limiting the risk of induction of local immune responses.[78,118,200]

Similar results were obtained in clinical trials for AAT deficiency and lipoprotein lipase (LPL) deficiency, in which vectors encoding the deficient enzyme where administered intramuscularly (Table 5). In the gene therapy trials for AAT deficiency, a mild increase in transgene expression levels was documented and persisted for the long term,[201,202] at least in subjects enrolled in the highest vector dose cohort (6×10^{13} vg).[202] AAV1 vector administration was associated with the development of anti-capsid T cell responses in all evaluated subjects.[202] Flotte et al.[203] reported a dose-dependent increase in AAT transgene levels in the circulation and a transient increase in creatinine kinase (CK) levels in most patients from the higher dose cohorts, and all patients developed humoral and T cell responses against the capsid. Although no anti-AAT antibodies were detected, cellular responses against the transgene product were documented, although the significance of the findings is unclear.[86,203] Despite the presence of cellular responses, transgene expression persisted at low levels up to 5 years in one patient from the highest dose cohort, leading to partial disease correction in that patient.[4] Persistent transgene expression seemed to be associated with the recruitment of Tregs into the muscle, and to the exhaustion

Table 5. Overview of Clinical Studies Based on Intramuscular AAV Injection

Disease	Sponsor	Phase	Study ID: ClinicalTrials.gov	Product	Dose	Immune Responses	References
Hemophilia B	Avigen	I/II	NCT00076557	ssAAV2-CMV-FIX	2×10^{11} vg/kg (n = 3) 6×10^{11} vg/kg (n = 3) 1.8×10^{12} vg/kg (n = 2)	pre-existing NAbs; dose-dependent humoral responses to AAV	118
ATT deficiency	University of Massachusetts	I	NCT00377416	AAV2-CB-AAT	2.1×10^{12} vg (n = 3) 6.9×10^{12} vg (n = 3) 2.1×10^{13} vg (n = 3) 6.9×10^{13} vg (n = 3)	pre-existing Nabs	201
	University of Massachusetts	I	NCT00430768	AAV1-CB-AAT	6.9×10^{12} vg (n = 3) 2.2×10^{13} vg (n = 3) 6.0×10^{13} vg (n = 3)	pre-existing NAbs, increase after injection; anti-capsid cellular responses in all evaluated participants	202
	Applied Genetic Technologies	II	NCT01054339	AAV1-CB-AAT	6.0×10^{11} vg/kg (n = 3) 1.9×10^{12} vg/kg (n = 3) 6.0×10^{12} vg/kg (n = 3)	pre-existing NAbs; cellular responses to the capsid in all participants; cellular responses to the transgene in two participants; detection of Tregs and exhausted T cells	4,86,203
LPL deficiency	Amsterdam Molecular Therapeutics	II	CT-AMT-010-01	AAV1-LPL^{S447X}	1×10^{11} vg/kg (n = 4) 3×10^{11} vg/kg (n = 4)	pre-existing NAbs; cellular responses to the capsid in four participants	39,204
	Amsterdam Molecular Therapeutics	II	NCT01109498 (CT-AMT-011-01)	AAV1-LPL^{S447X} (alipogene tiparvovec)	3×10^{11} vg/kg (n = 2) 3×10^{11} vg/kg + IS (n = 4) 1×10^{12} vg/kg + IS (n = 8)	pre-existing NAbs; anti-capsid cellular responses in nine participants	205
	Amsterdam Molecular Therapeutics	II/III	NCT00891306 (CT-AMT-011-02)	AAV1-LPL^{S447X}	1×10^{12} vg/kg + IS (n = 5)	pre-existing NAbs; anti-capsid cellular response in all participants; detection of Tregs and exhausted T cells	206,207,208
DMD	Nationwide Children's Hospital	I	NCT00428935	AAV2.5-CMV-minidystrophin	2.0×10^{10} vg/kg (n = 3) 1.0×10^{11} vg/kg (n = 3)	cellular responses to the transgene in four participants	73

IS, immunosuppression.

of effector T cells that expressed the inhibitory molecules PD-1 and PD-L1.[4,195,203]

In the context of LPL deficiency trials, a first study reported the intramuscular administration of an AAV1 vector produced in HEK293 cells,[18] carrying the sequence of a mutant version of the LPL enzyme (LPL^{S447X}). The vector was injected into eight patients divided in two dose cohorts of 1×10^{11} and 3×10^{11} vg/kg.[204] Despite the initial dose-dependent increase in LPL expression, the efficacy of gene transfer decreased in the long term with return to baseline levels after 18–31 months. The authors associated this effect with the presence of dose-dependent T cell responses against the capsid, which correlated with higher levels of anti-AAV1 IgG3 subclass antibodies, and with a transient increase in CK levels in plasma.[39,204] No antibody responses against the transgene product were detected. In view of these results, two new studies tested the efficacy and anti-capsid immune responses when administering the same vector, this time produced in a baculovirus system,[18] together with immunosuppression (Table 5).[205–207] All patients developed detectable cellular and humoral responses against the AAV capsid. Nevertheless, in the LPL trials[206,207,208] sustained transgene expression during the follow-up period was reported, together with the detection of Tregs and T cells with an exhausted phenotype, as reported in the AAT deficiency clinical trials[4,195] and in preclinical studies.[209]

These studies provide evidence that direct intramuscular delivery can elicit both inflammatory and tolerogenic responses in the local environment where the vector is delivered. This has also been elegantly modeled in mouse studies in which the role of Tregs in the regeneration of injured muscle was demonstrated,[210] possibly highlighting unique features of skeletal muscle when it comes to inflammation.

A different outcome was documented in a gene transfer trial for Duchenne muscular dystrophy (DMD).[73] In this study, lack of transgene expression in muscle was attributed to the expansion of cytotoxic T cells specific against epitopes found within the transgene product. Further studies showed the presence of preexisting $CD4^+$ and $CD8^+$ T cell-mediated immunity against the transgene product, which was detectable in 52.9% of DMD patients naive to gene transfer and was likely due to spontaneous dystrophin expression in revertant

fibers (Table 5).[73,74] The prevalence of anti-dystrophin immunity was decreased to 20.8% in patients receiving corticosteroid treatment.[74]

Based on these studies, it can be extrapolated that several factors can shape the immune responses in intramuscular gene transfer,[89] particularly when it comes to transgene-driven immunity. Furthermore, targeting all muscle groups in the body at therapeutically relevant doses is limited by this delivery route.

Intravascular and Systemic Delivery of AAV Vectors

Based on the early experience with intramuscular gene transfer, and on the need to target virtually the entire body to achieve therapeutic efficacy in many systemic neuromuscular disorders, research efforts in relatively recent years have focused on the systemic delivery of AAV vectors. Early studies in large animal models of hemophilia B and DMD showed that the intravascular delivery of AAV vectors was less immunogenic compared to intramuscular delivery, even in the absence of immunosuppression,[211] owing to the more evenly distributed transduction of muscle fibers, and possibly due to some levels of leaky tolerogenic expression of the transgene in the liver.[69,109]

Progress in AAV vector large-scale manufacturing helped to spearhead a number of gene therapy trials in which large vector doses were administered systemically to transduce the entire body. Among these, Audentes Therapeutics initiated a phase I/II study in infantile X-linked myotubular myopathy patients, consisting of systemic administration of an AAV8 vector coding for the *MTM1* gene under the control of a muscle-specific promoter given at doses up to 3×10^{14} vg/kg (ClinicalTrials.gov: NCT03199469).[185] Vector administration was well tolerated, and evidence of therapeutic benefit was documented. From an immunogenicity perspective, both transgene and capsid immune responses were detected, along with an increase in liver enzymes and in some cases increased creatinine kinase and troponin, possibly linked to some degree of systemic immune-mediated toxicity. All subjects enrolled in this trial received prophylactic corticosteroids.

Three additional studies of systemic AAV vector delivery for DMD currently ongoing are also worth mentioning because of specific challenges encountered. In at least two of the three trials, one sponsored by Solid Biosciences (ClinicalTrials.gov: NCT03368742) and another by Pfizer (ClinicalTrials.gov: NCT03362502), acute toxicities were experienced, possibly related to complement activation. There were no reports of such toxicities in a similar trial sponsored by Sarepta Therapeutics (ClinicalTrials.gov: NCT03375164). Also in these trials, large doses of AAV vectors were administered (in excess of 10^{14} vg/kg), and prophylactic corticosteroids were administered. In an attempt to modulate acute toxicities, the monoclonal antibody eculizumab was administered in some of these trials.

Overall, emerging data from systemic AAV vector delivery at high doses highlight both the remarkable therapeutic potential of the approach and unexpected, possibly immune-related toxicities. As publicly available data are somewhat limited at the time that this article was written, the etiology of the findings is not entirely clear, although they seem to concern multiple AAV serotypes across trials for various indications and tissue targets. Future studies and follow-up of subjects treated with high-dose AAV gene transfer will be useful to better understand the potential effect of the toxicities encountered on the safety and long-term efficacy of gene transfer.

Gene Transfer to Motor Neurons and the Central Nervous System

Systemic gene transfer to target motor neurons has been validated in the context of the clinical development of a gene therapy treatment for spinal muscular atrophy (SMA) that is marketed by AveXis-Novartis as Zolgensma. Efficacy of this approach was been initially shown in 15 infantile patients with mutations on both alleles of the *SMN1* gene and two copies of the *SMN2* gene. Patients were injected systemically with an AAV9 vector carrying the *SMN* gene under the control of a ubiquitous promoter.[186] Young pediatric patients were divided in two cohorts of 6.7×10^{13} and 2.0×10^{14} vg/kg. As in other systemic delivery studies, because most of the vector delivered systemically ends up in the liver, elevated liver transaminases were observed. Concomitantly, capsid-specific T cells were detected in peripheral blood and were attenuated by a course of prednisolone. Overall, four participants enrolled in this study showed transient elevation in transaminases. Based on the groundbreaking clinical results,[186,212,213] confirmed in a phase 3 trial (ClinicalTrials.gov: NCT03306277), the investigational gene therapy drug was recently approved.[214]

While there is little doubt about the potential therapeutic benefit of AAV-mediated gene transfer in the context of systemic neuromuscular diseases and in particular diseases affecting motor neurons, also in this context recent preclinical findings possibly related to vector immunogenicity are worth mentioning as potentially relevant. In a recent report,[215] toxicities associated with systemic delivery of high doses of AAV vectors encoding for SMN were observed in non-human primates (NHPs) and piglets. Liver enzyme elevation, including acute liver failure in one animal, was observed in NHPs. Dorsal root ganglia (DRG) sensory neuron lesions were also detected in both species, with a higher degree of severity in piglets. Toxicities described in this study appeared to be independent of capsid cellular immune responses. Similar symptomatic DRG degeneration was also described in a preclinical study conducted by AveXis-Novartis in NHPs following the intrathecal delivery of an AAV9-SMN vector,[216] prompting a hold on an ongoing trial of intrathecal vector delivery in SMA type 2 patients (ClinicalTrials.gov: NCT03381729). The nature of these preclinical findings is yet to be fully elucidated. To date, no similar toxicities were encountered in humans receiving the same AAV9-SMN vector systemically.[186] Several questions about these findings are still unanswered, including whether the observed toxicities are driven by the vector capsid, the transgene product, or both and whether they are in some way immune mediated. Of note, no symptomatic toxicities have been reported in an ongoing gene transfer trial for giant axonal neuropathy in which high doses of an

AAV9 vector are delivered intrathecally together with a course of immunomodulatory drugs (ClinicalTrials.gov: NCT02362438).

In contrast to systemic vector delivery, because of the physical separation to the systemic circulation mediated by the blood-brain barrier (BBB), brain delivery of AAV vectors has several peculiarities, including (1) that the cerebrospinal fluid (CSF) generally contains lower levels of antibodies able to neutralize AAV vectors compared to what is found in the general circulation; and (2) that the brain has a distinct subset of resident immune cells composed mainly of specialized macrophages called microglia, which, because of the lack of access to brain samples, are difficult to study. Immune responses in the human central nervous system (CNS) following gene transfer seem to be less frequent than in other body sites.[217] However, the tools we are using to track these responses are likely to be not sensitive enough to detect, for example, local asymptomatic, discrete responses that could lead to gradual clearance of transduced cells.

Pre-existing NAbs to AAV do not appear to be an issue in the context of direct AAV vectors into the CNS. For instance, it has been shown that the presence of circulating anti-AAV-NAbs up to a 1:128 titer had no inhibitory effect on efficacy of the AAV9-GFP gene transfer when delivered to the CSF in NHPs.[218] Conversely, the concentration of AAV9-NAbs in circulation increases post-vector infusion, both systemically and in the CSF.[219]

As mentioned, the brain possesses specialized resident immune cells that are yet to be studied in terms of their response to the AAV vectors. Microglia are initial responders to pathogens or tissue damage and are responsible for initiating an inflammatory response in brain. Alternatively, while phagocytosing dead or dying cells, microglia actually prevent the release of pro-inflammatory signals from necrotic tissue and thus limit further brain damage.[220] Additionally, microglia can also phagocytose viable cells that were infected by a virus, as a consequence, for example, of the intracellular calcium dysregulation and phosphatidylserine externalization observed in the context of adenovirus infection.[221] However, it is not certain whether transduction of neurons by AAV vector could elicit levels of cellular stress that can lead to clearance of transduced cells by microglia.

The infiltration of microglia to inflammation sites can be followed in tissue sections by fluorescent microscopy detecting ionized calcium binding adaptor molecule 1 (Iba1)-positive cells. The activation state can be inferred from the cellular morphology, where a resting microglial cell has elongated ramified processes that rapidly retract upon recognition of a pathogen or other inflammatory stimuli. Microglial cells will, in these circumstances, become a mobile effector cell.[222] Obviously, this type of analysis is not feasible for the purpose of tracking immune responses during clinical trials, which are commonly limited to testing anti-capsid and anti-transgene antibodies and T cells in peripheral blood or CSF.

Direct delivery of AAV vectors into the brain causes little or no response as measured by IFNγ ELISPOT in PBMCs from infused subjects (reviewed elsewhere[223]). One important feature to consider in this particular clinical setting is that the doses of AAV vectors administered thus far were relatively small. Nevertheless, a direct injection in brain can also be associated with a local inflammatory response. Similar to muscle, systemic infusion of AAV vectors into the CSF may decrease vector immunogenicity, although, because it requires higher vector doses, it does increase systemic vector exposure. For example, in a mouse model of Niemann-Pick disease type A (NPD-A), it was shown that the delivery of AAV9-ASM to the CSF through the cerebellomedullary (CM) cistern led to transgene expression within the central and peripheral nervous systems. When compared to the direct intracerebellar injection, this route of delivery did not trigger inflammation.[224] Similarly, administration of AAV9 vectors into the CSF of dogs resulted in widespread transduction of the brain,[219] with also leakage of the vector into the systemic circulation and transduction of the liver.

Across the ongoing trials, the approaches to modulation of potential immunogenicity associated with gene transfer in the CNS vary significantly, ranging from concomitant use of combination of immunosuppressive drugs (ClinicalTrials.gov: NCT02362438, NCT03612869) for an extended period to no immunosuppression (EudraCT: 2015-000359-26). A published example comes from a clinical trial in subjects with Canavan disease with mutations on the gene encoding for aspartoacylase (ASPA) who received an AAV2-ASPA vector infused intracranially.[225] No immunosuppression was used during this trial, and only 3 out of 10 subjects developed low to moderately high levels of AAV2 NAbs in blood compared to baseline. In another trial, patients with metachromatic leukodystrophy (MLD) were administered AAVrh.10-arylsulfatase A (ARSA) by intracerebral injection (ClinicalTrials.gov: NCT01801709). A short course of steroids was used to avoid potential immune reactions starting 1 day prior to the vector infusion and given for 10 days. Anti-capsid antibodies were detected in blood and CSF of all patients. Only one out of four participants developed anti-ARSA antibodies detectable in blood but not in CSF (C.G. Bonnemann, 2019, National Center for Advancing Translational Sciences/NIH, conference). Another example of immune suppression combined with the CNS gene transfer may be illustrated by the AAV9-mediated delivery of gigaxonin for cross-reactive immunologic material (CRIM)-negative giant axonal neuropathy patients (ClinicalTrials.gov: NCT02362438). To mitigate the risk for CRIM-negative participants to mount an immune response against the transgene, an immunosuppressive regimen including overlapping dosing of methylprednisolone, prednisone, tacrolimus, and rapamycin was used. The approach was successful in preventing inflammation, as in immunosuppressed participants no pleocytosis was detected. After vector infusion, anti-AAV9 NAbs appeared in blood and CSF. Of note, an IFNγ ELISPOT assay demonstrated the presence of AAV9-specific, but not gigaxonin-specific, T cells circulating in blood in subjects dosed with the vector and corticosteroids alone (C.G. Bonnemann, 2019, National Center for Advancing Translational Sciences/NIH, conference).

Conclusions

The gene therapy field is experiencing one of its most exciting periods. Long-term efficacy has been achieved in several clinical trials, and gene therapy drug candidates are reaching late-stage clinical development and market approval. Diseases previously untreatable or for which suboptimal treatments were available are now being cured, delivering previously unimaginable outcomes to patients. Since the first reports of immune-related toxicities associated with AAV vector administration, much progress has been made in understanding the interactions between viral vectors and the host immune system. Yet, new unforeseen complexities are still emerging from trials in which high vector doses are administered systemically. This is clear evidence that many questions about AAV vector immunogenicity are still unanswered.

Are All AAV Vectors the Same?

Experience from clinical trials and preclinical models has helped to guide the design of the ideal AAV vector. The best trade-off one can currently imagine is to engineer AAV vectors with better transduction efficiency, carrying optimized therapeutic transgenes and with reduced immunogenic profiles (CpG-depleted genome,[66] immunologically inert capsids, vector preparations with low levels of contaminants, minimum amounts of empty capsids[226]). The ideal vector then would provide a high therapeutic index, as it would permit therapeutic efficacy at doses sufficient to bypass pre-existing humoral immunity, but not above the threshold of activation of T cell-mediated immunity. While certainly the appetite of the field for novel AAV capsids is still high,[227] from an immunological standpoint, with the exception of the potential for NAb escape,[228] it is still unclear what are the critical parameters that would make a capsid less likely to trigger immune-mediated toxicities. The fact that AAV vectors used in clinical trials are manufactured with different platforms[18] further complicates the analysis of the emerging data.

Are There Any Preclinical Models that Are Useful to Study Anti-AAV Cellular Immune Responses?

Small[33,226] and large[31] animal models have been useful in studying the impact of anti-AAV humoral responses and devising strategies around them.[119] However, the onset of anti-capsid cellular responses observed in several clinical trials has never been observed before in any of the preclinical animal models employed, even in those susceptible to natural AAV infections such as NHPs,[31,229] possibly due to differences in the T cell compartment when compared to humans.[43,230] The lack of relevant animal models remains therefore an important hindrance to fully understanding the biology behind AAV vector immunogenicity.

The first attempts to generate a model via immunization of mice against the AAV capsid were unsuccessful.[231–233] More recently, few mouse models have been established,[52,234,235] along with *in vitro* human systems.[42,60,64] Although promising, as these models contributed to a better understanding of AAV vector immunogenicity, to date, the efficacy strategies to modulate capsid immune responses can be only tested in the clinic. Animal models, in particular NHPs, remain important to test the safety of any immunomodulatory regimen to be used in combination with AAV gene transfer.[91]

Are Circulating T Cells Reflective of Immune Responses in Target Organs?

Circulating T cells are routinely utilized for immunomonitoring in AAV trials (*vide infra*). However, detection of T cell reactivity has not always been associated with clinical evidence or immune-mediated toxicities, indicating that observations made in the periphery might not accurately reflect the local immunological events taking place in the target tissue, where capsid antigens are being presented. While for obvious reasons T cell responses in peripheral blood remain an important surrogate marker to track vector-related immune responses, analysis of *in situ* immune responses might help refine our understanding of the mechanisms by which loss or maintenance of transgene expression may occur. Sample collection is likely to be challenging, as clinical manifestations of immune responses in clinical trials have been quite variable in terms of timing, and because often the biomarkers used to monitor these responses are not fully reliable.

What Is The Role of Pre-existing Immunity to WT AAV and Its Interplay with Adaptive Immune Responses to AAV Vectors In Humans?

It is well established that humans carry both humoral and cell-mediated immunity to WT AAV (*vide supra*). These responses are usually found more frequently in adults, while children are frequently immunologically naive to AAV. Whether the T cell responses observed in AAV gene transfer trials were primary or memory recall responses has been a matter of debate, as often the delay between vector administration and detection of T cell activation is incompatible with what is expected for a re-exposure to an antigen encountered in the past. Monitoring immune responses across trials and correlation with the history of exposure to AAV may help in the future to clarify this point. Notably, the recent findings in DMD gene transfer trials, in which complement activation was documented, may be related to the presence of anti-AAV-binding antibodies prior to vector infusion. Thus, in the context of these preliminary observations, it could be informative to carefully assess the history of pre-exposure to WT AAV in relationship to the outcome of gene transfer. Similarly, important information could be gathered by studying what is the effect of activation of innate immune responses triggered by vector administration on memory T cells that originated from exposure to WT AAV.

Should We Worry About Transgene-Specific Immune Responses and How They Might Impact the Onset of Anti-AAV Immune Responses?

Despite the fact that a wealth of preclinical data are available on immune responses to the transgene product in AAV gene transfer, relatively little information is available on whether this knowledge would faithfully translate to the clinic. Several groups showed that delivery of AAV vectors to the liver induces transgene-specific tolerance,[80,81,85,105] and liver gene transfer has also been used in

hemophilia A and B dogs to eradicate anti-F.VIII and F.IX neutralizing antibodies, respectively.[76,77] Although these preclinical data on liver tolerance are highly convincing, the open question is whether this concept will reliably translate to humans.

Thus far, no transgene immune responses have been detected in liver trials, although most of the enrolled subjects in these studies were at low risk of counting an anti-transgene immune response. Conversely, transgene-specific immune responses have been detected in trials of CNS gene transfer,[88] eye,[131] and muscle-directed gene transfer.[73,185] Immunosuppression, vector engineering,[79] or simply the use of alternate strategies to deliver a therapeutic transgene to a potentially pro-tolerogenic tissue[81] are all potential solutions to the issue of transgene immunogenicity. Importantly, the long-term impact of transgene immunity needs to be carefully assessed, particularly on terms of clinical outcomes.[86]

In clinical trials, anti-transgene immunity will require careful evaluation in patients with pre-existing immunity toward the transgene product, either resulting from the underlying disease[73,74] or to previous treatment with recombinant protein.[71] Moreover, some diseases involving lysosomal[236] or metabolic[75] alterations may present with alterations of the immune system, which may interfere with gene transfer.

What Are the Most Promising Approaches to Immunomodulation in AAV Gene Transfer?

Immunomodulation is broadly used in the context of gene transfer to reduce AAV vector immunogenicity, and eventually to allow for repeated interventions. Corticosteroids have been administered in most trials, and they certainly have helped to modulate immune-mediated toxicities and achieve long-term transgene expression. However, in some cases the administration of more complex regimens was required to manage capsid or, potentially, transgene immunogenicity. For vector redosing, several strategies to prevent anti-AAV antibody production have been proposed, with only one tested in the clinic to date (ClinicalTrials.gov: NCT02240407). While some preclinical results are promising, it is clear that pre-existing B cell immunity is a major hurdle, due to the fact that targeting a primed immune system is much harder than simply preventing an immune response to occur.

It is early to identify which strategy or combination of strategies will be the best in the clinic; however, a few general considerations can be made:

- The ideal immunomodulatory regimen has to offer an acceptable risk-benefit profile, particularly in the context of a given disease indication and patient population (e.g., not all drugs are approved for pediatric use).
- A careful evaluation of drug interactions should be made to ensure that the co-administration of immunomodulatory drugs does not interfere with the safety and efficacy of gene transfer (e.g., some drugs can interfere with transgene tolerance induction).
- The ideal immunomodulatory regimen for gene transfer is simple and transient. A short course of clinically approved immunosuppressive drugs may offer an easy path to the clinic and modulate effectively vector-related immune-mediated toxicities.
- "One size fits all" is unlikely to apply to gene transfer with AAV vectors. Depending on the vector dose, target tissue, disease indication, patient population, and other factors, tailored immunosuppressive regimens will likely have to be identified.

Building Best Practices in Immunomonitoring

As many questions remain on AAV immunogenicity, the field of AAV gene therapy research needs further efforts to resolve the complexity of capsid-related immune responses. The harmonization of patient monitoring using standard guidelines and quality controls, to check immune assay performance over time and across clinical trials, would greatly facilitate the comparison of data, and subsequently the understanding of the complexity of anti-AAV immune responses. Additionally, aside from monitoring IFNγ activation, additional markers of T cell activation, such as TNF-α,[42] which better reflect the profile of activation of T cells specific to the AAV capsid in humans, should be routinely reported.

The years to come will continue to bring forward a wealth of preclinical and clinical data that hopefully will provide precious insights on some of the questions we outlined in this review. Gaining a more sophisticated understanding of the AAV vector technology, as well as the nuances of the interactions between AAV vectors and the host immune system, will likely provide a path forward to further extend the success of this still novel and highly promising therapeutic paradigm.

AUTHOR CONTRIBUTIONS

H.C.V., K.K., and F.M. wrote the manuscript.

CONFLICTS OF INTEREST

F.M. and K.K. are employees of Spark Therapeutics. H.C.V. declares no competing interests.

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