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Review

Immune Response Mechanisms against AAV Vectors in Animal Models

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Early preclinical studies in rodents and other species did not reveal that vector or transgene immunity would present a significant hurdle for sustained gene expression. While there was early evidence of mild immune responses to adeno-associated virus (AAV) in preclinical studies, it was generally believed that these responses were too weak and transient to negatively impact sustained transduction. However, translation of the cumulative success in treating hemophilia B in rodents and dogs with an AAV2-*F9* vector to human studies was not as successful. Despite significant progress in recent clinical trials for hemophilia, new immunotoxicities to AAV and transgene are emerging in humans that require better animal models to assess and overcome these responses. The animal models designed to address these immune complications have provided critical information to assess how vector dose, vector capsid processing, vector genome, difference in serotypes, and variations in vector delivery route can impact immunity and to develop approaches for overcoming pre-existing immunity. Additionally, a comprehensive dissection of innate, adaptive, and regulatory responses to AAV vectors in preclinical studies has provided a framework that can be utilized for development of immunomodulatory therapies to overcome or bypass immune responses and for developing strategic approaches toward engineering stealth AAV vectors that can circumvent immunity.

Adeno-Associated Virus

Adeno-associated virus (AAV) is a single-stranded DNA dependovirus and member of the parvovirus family. The wild-type genome of AAV is 4.7 kb coding for replication (rep) and structural (cap) proteins. AAV infection is not associated with any disease in humans and other mammals, which are natural hosts for AAV, and the wild-type virus is weakly immunogenic. However, AAV replication is dependent on immunogenic helper viruses that promote inflammation, resulting in humoral and cell-mediated immune responses directed against the AAV capsid proteins. Thus, from natural infection, humans may have pre-exiting immunity with antibodies and immunological memory against the AAV capsid.

AAV as a Gene Therapy Vector

AAV vectors are generated by replacing the rep and cap genes with a transgene expression cassette, while retaining the flanking *cis* viral inverted terminal repeats (ITRs).[1] Capsids from different AAV serotypes, natural or engineered, can be used to cross-package AAV genomic DNA with AAV2 ITRs to direct vector tropism to a target tissue or organ.[1] The AAV vector genome can be packaged as single-stranded (ssAAV) DNA, similar to wild-type AAV or self-complementary (scAAV) with double-stranded DNA.[1] The viral capsid is made up from three proteins VP1, VP2, and VP3, in which VP2 and VP3 are shortened versions of VP1. Thus, the capsid proteins and transgene product constitute the only immunological antigens. However, since the viral capsids are derived from wild-type AAVs, AAV vectors can be recognized by pre-existing adaptive immune responses.

Clinical Experience with AAV Vectors

Presently, there are two FDA approved AAV biologicals for the treatment of inherited blindness (Leber's congenital amaurosis) and spinal muscular atrophy (SMA).[2,3] Additionally, there are a substantial number of AAV gene therapy clinical trials evaluating therapeutic efficacy for a number of diseases. However, despite the accelerated use of AAV in clinical studies, there have been repeated reports of toxicities that have compromised transgene product expression.[4–6] In some studies, immune responses against either the AAV capsid or transgene product have been identified as contributing to the reduction or complete loss of expression. Despite the fact that AAV vectors have a small immunological footprint, infection of humans with wild-type AAV and cross-reactive responses to different AAV serotypes poses a risk for sustained transgene expression. Further, the use of ever-higher vector doses in clinical trials may reveal new toxicities and require reevaluation of current immune suppression protocols.

Innate Immune Responses to AAV Vector

Innate Immunity

Host innate immunity recognizes and rapidly responds to microorganisms and pathogens through recognition of pathogen-associated molecular patterns (PAMPs), common shared structural features found on microorganisms and pathogens.[7–9] These PAMPs are recognized by pattern recognition receptors (PRRs), largely expressed

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on professional antigen-presenting cells (APCs), which are critical cells for linking innate and adaptive immune responses.[7–9] Toll-like receptors (TLRs) are the most studied PRRs and are strategically localized on the cell surface or intracellularly in the endosome for early detection of invading pathogens. The endosomal TLRs (TLR3, TLR7, TLR8, and TLR9) recognize viral nucleic acids (ssRNA, dsRNA, ssDNA, and dsDNA), respectively, typically following receptor-mediated endocytosis of virus. For example, the unmethylated CpG DNA viral genome of a DNA virus, such as an AAV and AAV vectors, is sensed by TLR9 and leads to the activation of an anti-viral immune response mediated by the release of type I interferons (IFNs) and induction of a T helper 1 (Th1) adaptive immune response. The surface TLRs (TLR2, TLR4, TLR5, and TLR6) typically recognize extracellular microorganisms and trigger host innate immunity through acute phase, neutrophil, and other pro-inflammatory responses and largely shape a Th2 adaptive immune response. Many pathogens are sensed by both surface and endosomal TLRs. Proposed innate ligands of AAV vectors include the AAV capsid,[10] CpG containing AAV genome,[11] and dsRNA.[12]

Innate Recognition of AAV Vectors

A comparative study of immune responses to AAV and adenovirus (Ad) vectors provided early awareness that AAV vectors could trigger innate immune responses in mice.[13] However, pro-inflammatory cytokine responses to AAV vectors was transient with a pronounced response 1 h post intravenous (i.v.) injection but returned to baseline 6 h post injection. Liver infiltration of neutrophils and macrophages showed a similar time course, and liver necrosis was only evident in Ad vector-treated mice.[14] The differential response between AAV and Ad may be attributed, in part, to differences in their genomes as Ad is a dsDNA virus, which is a more effective TLR9 agonist than the ssDNA AAV vector genome.[15] AAV infection of plasmacytoid dendritic cells (pDCs) and conventional dendritic cells (cDCs) isolated from mice deficient in TLR9 and myeloid differentiation primary response protein 88 (MyD88) and not TLR2 or Trif proteins had significantly reduced secretion of type I IFNs specifically in pDCs.[11] This activation was independent of both capsid serotype and transgene. DNase treatment of vector confirmed that the AAV genome was the primary TLR9 ligand. Further, adaptive immune responses to intramuscular (IM) delivery of an AAV vector and the transgene product were shown to be dependent on innate sensing by TLR9, MyD88, and type I interferon receptor (IFNR).[11] Innate immune responses in the liver were investigated following i.v. ssAAV and scAAV vector administration in mice.[16] Activation of pro-inflammatory genes was shown to be TLR9 dependent and was observed only with scAAV vectors, independent of capsid serotype.[16] The innate response was rapid, appearing within 2 h after gene transfer, and transient, resolving 9 h post vector.[16] Of note, of the different PRRs measured, mRNA levels of TLR9 was significantly upregulated only with scAAV vectors along with a mild elevation in TLR2. Scoring of liver inflammation revealed that increased levels of neutrophils, macrophages, and natural killer (NK)-cells were only observed in mice infused with a scAAV vector and could be prevented by co-administration of a TLR9 antagonist.[16]

Do the dsRNA-Mediated Responses Play a Role Innate Immunity to AAV Vectors?

The generation of dsRNA following AAV gene delivery and activation of cytoplasmic RIG-1/MDA5 innate sensors was proposed as an alternative explanation for immunotoxicities observed in clinical trials.[12] This study hypothesized that bidirectional transcription facilitated by the inherent promoter activity of the AAV ITRs could generate negative-strand RNAs and trigger a dsRNA-mediated innate immune response. Using a scAAV-hFIX-opt vector, gene expression of MDA5, RIG1, and interferon-β (IFN-β) and transduction was assessed at 4 and 8 weeks in mice with humanized livers—mouse livers partially reconstituted with human hepatocytes. A mild ~2-fold increase in gene expression of MDA5 and RIG1 and a less than 2-fold drop in transduction was reported.[12] Of note, a significant increase in IFN-β expression was reported at 4 weeks in two animals and at 8 weeks in one animal. The significance of these results is somewhat limited by the small number of immune-deficient mice studied and may not reflect responses in immune competent mice. It should be noted that the proposed role of ITR promoter activity in the generation of dsRNA is somewhat controversial and that it can result in inconsistent innate activation. A recent study found that co-administration of a TLR7 agonist (to mimic dsRNA sensing) along with IM delivery of a ssAAV vector failed to reduce transgene product levels in mice, raising doubts about whether dsRNA sensing is relevant in priming AAV vector and transgene product adaptive immune responses.[17] Thus, the role of dsRNA in the overall innate sensing of AAV vectors remains unclear and requires further study.

Innate Recognition Shapes Adaptive Responses to Capsid and Transgene Product

Co-administration of TLR2, TLR4, TLR7, or TLR9 ligands with IM delivery of an AAV vector was studied to determine the impact on adaptive immune responses to both AAV vector and transgene product. Mice receiving a TLR9 agonist along with IM delivery of ssAAV-*F9* vector were the only group to have a transient increase in factor IX protein (FIX) antibodies.[17,18] Overall, these data show that TLR9 is critical in forming adaptive responses following AAV vector administration. Complimentary studies in mice deficient in intrinsic components of innate recognition and activation ($TLR2^{-/-}$, $TLR4^{-/-}$, $TLR7^{-/-}$, $TLR8^{-/-}$, $TLR9^{-/-}$, and $MyD88^{-/-}$) have revealed critical sensors and immune cells needed for Th1 antibody responses to AAV vectors.[19,20] In terms of capsid antibody responses, the only difference noted was a deviation from a Th1 to Th2 capsid antibody response in $MyD88^{-/-}$ mice independent of delivery route (IM or i.v.). Whereas both TLR9 and MyD88 were shown to affect transgene product-specific T cell adaptive responses.[19]

Summary of Preclinical Studies on Innate Immune Responses to AAV Vectors

TLR-specific and downstream innate signaling knockout mice have been essential in defining critical PRRs that sense AAV vectors. In mice, a scAAV vector is required to induce a measurable innate immune response to AAV vector delivery to the mouse liver,[16] despite clear evidence of adaptive immune responses in ssAAV-treated

human patients. Moreover, even though these immunological models have demonstrated inflammatory, humoral, and adaptive responses, there is often no change in the levels of the transgene product. For example, in C57BL/6 mice, co-delivery of a TLR9 agonist with IM AAV vector delivery was needed to impact transgene-specific adaptive immune responses,[17,18] whereas in other strains, IM AAV vector delivery alone is sufficient for inducing adaptive immune responses. Despite these limitations, murine studies on innate immunity to AAV vectors have revealed an important role for TLR9-MyD88 recognition and signaling in activating inflammatory responses and modulating adaptive responses to both AAV vectors and their transgene products. However, there have been limited reported studies in large animal models on innate sensing of AAV vectors. Recent studies in non-human primates (NHPs) have reported innate immune responses following administration of AAV vectors into the immune privileged eye.[21,22]

Adaptive Immune Responses to AAV Vector

Linking Innate and Adaptive Immunity

Innate immunity is a critical component to an effective adaptive immune response, which includes humoral immunity (B cells) and cell-mediated immunity ($CD8^+$ T cells).[9] Unlike innate immune cells, adaptive immune cells possess antigen-specific receptors generated through recombination and somatic hypermutation and are capable of recognizing a vast repertoire of antigens.[23] Activated APCs upregulate cell surface expression of co-stimulatory molecules, major histocompatibility class II molecules (MHC II), and release inflammatory cytokines, which lead to activation of both B and T cells.[23] $CD4^+$ T helper cells play a central role in enhancing adaptive immunity by providing additional co-stimulatory signaling and cytokines to aid in the maturation and class switching of B cells, activation of $CD8^+$ T cells, and immunological memory.[23] Thus, strategies preventing the activation of APCs or $CD4^+$ T helper cells have become a major focus for preventing AAV vector immune responses.

Pre-existing Immunity to AAV

AAV and associated parvoviruses naturally infect mammals and thus, investigators should be aware of the impact of pre-existing adaptive immune responses, capsid neutralizing antibodies (NAbs), and memory capsid $CD8^+$ T cells when designing their studies.[24–26] Further, patients with pre-existing NAbs are presently excluded from clinical trials and this can range from 20%–70% of eligible patients depending on region and capsid serotype.[27] However, capsid T cell responses were not considered to be prohibitive until immunotoxicities were identified in clinical trials,[4,6] as animal studies did not predict this outcome.[6] NHPs with AAV8 pre-existing NAbs effectively block AAV8 liver gene transfer[28] and often these NAbs can cross-react with other capsid serotypes.[29] Thus, it is important to consider the impact of pre-existing AAV immunity in NHPs when designing and reporting studies, particularly for those investigators outside of the gene therapy field.[30] AAV9 NAbs can inhibit intrathalamus delivery of an AAV9 vector in mice suggesting that even direct tissue injection into the immune privileged CNS may not avoid pre-existing NAbs.[26] Studies in NHPs revealed that memory capsid $CD8^+$ T cells, derived from wild-type AAV8 infection, were unable to eliminate AAV8 transduced hepatocytes[28] in contrast to observations in human clinical trials.[4] Immunological profiling showed that both $CD4^+$ and $CD8^+$ capsid reactive T cells in NHPs displayed functional and phenotypic differences as compared to humans.[31]

AAV Capsid NAbs and Screening

Despite transiently activating innate immunity,[13] natural infection with AAV and injection of AAV vectors results in NAb formation. Since both neutralizing and non-neutralizing capsid antibodies are formed, detection of antibodies by ELISA alone is uninformative[32] and functional NAb assays have become a standard for screening patients. Interestingly, non-neutralizing antibodies may positively impact gene transfer as one study demonstrated that antibody binding initially increased liver transduction of an AAV8 vector and enhanced vector genome copy number in the liver compared to controls.[32] AAV NAb assays are typically conducted using serial dilutions of the test plasma with the appropriate AAV test vectors *in vitro* on a target cell line or *in vivo* using passive immunization of the test plasma into mice.[26,33–36] Recently an alternative *in vitro* method was published that measures AAV cell binding inhibition by qPCR.[37] However, these assays have their inherent benefits and limitations.[26,38] At present, there is no standardized protocol used to conduct *in vitro* NAb assays, making it challenging to compare results among different investigators. Alternatively, several studies present some limitations to *in vivo* Nab assays such as reduced sensitivity to *in vitro* testing,[33] not mimicking *in vivo* generated NAb,[26] and not being effective at high throughput screening.[38]

Mechanisms and Prevention of Capsid NAbs

AAV vector-derived capsid NAbs are dependent on route of vector administration[39] and viral particle uptake by APCs and presentation to $CD4^+$ T cells.[40,41] In mice, complement interaction with AAV vector particles facilitates macrophage uptake and activation.[42] *In vitro* studies with human PMBCs from wild-type AAV-infected donors showed a role for interleukin-1β (IL-1β) in B cell maturation and capsid NAb production, which was confirmed in mice using a neutralizing IL-1β antibody.[43] Several studies in knockout mice have shown that TLR9 may or may not be dispensable for capsid Nab formation, whereas loss of downstream signaling mediator MyD88, particularly in B cells, significantly reduced NAb titers and shifted antibodies from a Th1 (IgG2c) to a Th2 (IgG1) immune response.[11,19] Prophylactic immune modulation has been shown to prevent capsid NAb through a variety of B and T cell targeted therapies including: downregulation of CD4 on T cells with the antioxidant MnTBAP,[44] a non-depleting ND-CD4 antibody,[40] B cell depletion with anti-CD20, or rapamycin nanoparticles.[45] However, the duration and choice of immune suppression regimen can impact the effectiveness of preventing capsid NAbs. In NHPs, tacrolimus and mycophenolate mofetil (MMF) failed to completely prevent capsid NAbs, with one animal showing an elevated NAb titer following cessation of immune suppression.[28] A combination of MMF, anti-thymocyte immunoglobulin, methylprednisolone, tacrolimus, and rituximab over a course of 12 weeks prior to and following i.v. delivery of an AAV5 vector showed control of capsid

antibodies during immune suppression with elevation following cessation of immune suppression.[46]

Circumvention of Capsid NAbs

Early studies in NHP with pre-existing AAV8 NAbs and mice with an *in vivo* passive immunization assay demonstrated that low AAV NAb titers can prevent liver transduction following i.v. AAV8 vector delivery.[36] Additionally, studies in mice revealed slow circulatory clearance rates of AAV2 or AAV8 vectors,[47] potentially increasing the risk for neutralization. Temporary removal of anti-AAV capsid antibodies by saline flush of the portal vein,[48] plasmapheresis,[49] and plasmapheresis with immunoadsorption[50] has proven effective for improving transduction. *In vivo* administration of a proteasome inhibitor (PI), bortezomib, eliminated hypersensitive antibody-producing plasma cells and significantly reduced AAV Nab titers for vector re-administration.[51] Alternatively, AAV8-*cF8* treated hemophilia A dogs demonstrated a significant reduction in AAV8 NAb titers ~8 years after initial dosing of vector with bortezomib, allowing for vector re-administration.[52] Immune suppression with rituximab (anti-CD20) and cyclosporine A (CsA) was tested in NHPs following i.v. delivery of an AAV8 vector; however, this failed to significantly reduce AAV8 Nab titers, but investigators reported a reduction in cross-reactive AAV6 NAbs that allowed for vector re-administration.[53]

Vector modifications including the use of alternative serotypes,[54,55] empty decoy capsids,[56] and exosome enveloped AAV vector particles[57] have also shown success in animal studies avoiding pre-existing capsid NAbs. However, flexibility may be limited for switching to an alternative serotype because of cross-reactive neutralizing capsid antibodies. While studies in mice and NHPs[56] and humans[58] were conducted with formulated vector preps containing empty decoy capsids, such excessive capsid loads may lead to immunotoxicities.[5] Alternatively, direct tissue injection, such as IM delivery, may bypass pre-existing capsid NAb, but this may increase the risk of transgene immunity[59] and may not always work.[26]

Animal Models to Address AAV5 Vectors Resistance to NAbs

Since AAV5 is the most structurally divergent of the naturally isolated AAV capsids, it was hypothesized to have a reduced risk of neutralization, both directly and indirectly by avoiding cross-reactive NAbs. Therefore, several groups have developed AAV5-based vectors for clinical trials including hemophilia A and B and acute intermittent porphyria.[60–62] However, there has been some controversial results published regarding NAbs to AAV5. One group evaluating an AAV5 vector for hemophilia B therapy presented data that AAV5 vectors can successfully transduce liver in the presence of NAbs using plasma from clinical trial participants and NHP studies.[63] More recently, another group evaluating an AAV5 vector for hemophilia A gene therapy reported that 4 of 5 NHPs positive for capsid antibody (TAb^+) and transduction inhibition (TI^+) had a considerable reduction in circulating human FVIII levels compared to Tab^- and TAb^- TI^+ animals.[64] The data demonstrate that lack of standardization and reliance on *in vitro* assays makes it challenging to compare results and predict *in vivo* outcomes.

Cytotoxic T Lymphocyte Response to Capsid

Since first described in humans, there still remains no suitable animal model to predict the immunotoxicities observed in patients.[5,38] In murine models, capsid $CD8^+$ T cells can be induced following IM and i.v. injection of an AAV vector.[65–68] One hypothesis proposed that capsids dependent on heparin for cell entry are acquired by DCs and presented to capsid T cells.[69] Despite data supporting this hypothesis in both mice and NHPs,[69] this was not observed in humans.[70] A surrogate capsid epitope, the immunodominant H-2Kb ovalbumin epitope SIINFEKL, was introduced into the AAV capsid to better track responses with epitope-specific reagents. Studies in C57BL/6 mice showed that AAV2-SIINFEKL and AAV8-SIINFEKL vectors activated capsid $CD8^+$ T cells and could induce proliferation of adoptively transferred dye labeled OT-I $CD8^+$ T cells, $CD8^+$ T cells from mice transgenic for the SIINFEKL MHC-I restricted T cell receptor (TCR).[71–73] However, despite showing that capsid $CD8^+$ T cells could kill peptide-pulsed target cells *in vivo*, these cells failed to eliminate AAV-SIINFEKL transduced hepatocytes.[65] Further, studies in mice focusing on the generation of capsid memory $CD8^+$ T cells and their subsequent activation following AAV liver gene delivery also failed to induce clearance of AAV transduced hepatocytes.[74]

IM injection of an AAV2 or AAV6 vector in wild-type outbred dogs resulted in a robust T cell immune response to capsid that was independent of transgene and promoter.[75] A more recent study found evidence of a capsid $CD8^+$ T cell response in a hemophilia A dog retreated with a second dose of an AAV8-*cF8* vector with mild elevation in ALT.[52] However, it is unclear if the interferon-gamma (IFN-γ)-secreting capsid $CD8^+$ T cells detected at day 17 were related to the loss in transgene as inhibitory anti FVIII antibodies were detected at day 59 post vector, likely explaining the loss in coagulation activity.[52] Long-term analysis of i.v.-injected NHPs heterozygous for $LDLR^{-/+}$ with an AAV8-*hLDLR* vector showed a wide range of responses including capsid $CD8^+$ T cells in peripheral blood, liver, spleen, and bone marrow.[76]

A new mouse model was developed based on the *in vivo* and *in vitro* expansion of capsid reactive $CD8^+$ T cells using the $H2\text{-}L^d$ native immunodominant capsid epitope shared by both AAV2 and AAV8 capsids, as well as human HLA-B*0702.[77] Adoptive transfer of these expanded and activated capsid $CD8^+$ T cells into AAV transduced immune-deficient recipient mice demonstrated specific hepatocyte killing with transient transaminitis.[77] *In vivo* administered PI or engineered capsids with reduced capsid epitope presentation suppressed capsid-specific $CD8^+$ T cell elimination of hepatocytes.[77] Follow-up studies demonstrated that an immune-deficient recipient was not necessary,[78] possibly allowing for the generation and testing of capsid $CD8^+$ T cells within the same animal. However, mouse-based models are presently not able to evaluate human hepatotropic capsids[79] and humanized mice with human immune systems and livers require further development.[38,80]

Cross-Presentation of AAV Capsid

Classic MHC-I presentation by APCs is restricted to endogenously synthesized and proteasomal degraded proteins. However, certain

APCs have an alternative pathway, cross-presentation, that allows MHC-I presentation of epitopes derived from exogenous proteins, such as viruses or pathogens, which are normally relegated to MHC-II presentation. Cross-presentation of AAV capsid and primary activation of capsid-specific $CD8^+$ T cells has been discussed as an alternative hypothesis to the activation of memory capsid $CD8^+$ T cells and immunotoxicity.[38] Several murine studies support that cross-presentation of AAV vectors plays a role in activating cellular immunity against the AAV capsid.[6,65,68] Capsid $CD8^+$ T cell responses were shown to be significantly diminished in *TLR9*$^{-/-}$, *MyD88*$^{-/-}$, and *IFNR*$^{-/-}$ mice, suggesting an important role for pDCs and type I IFN in activating cell-mediated immunity to AAV vectors.[11] Mechanistic studies in mice revealed a co-operative role between plasmacytoid pDCs and cDCs for inducing a primary $CD8^+$ T cell response directed against the capsid.[65] These studies demonstrated that cross-presentation of capsid-derived epitopes to $CD8^+$ T cells was dependent on TLR9 sensing of the AAV vector genome by pDCs and subsequent licensing of cDCs mediated by release of type I IFN from pDCs.[65]

Animal Model to Evaluate the Effectiveness of Prednisone on Capsid CD8⁺ T Cell Responses

Despite extensive use in recent AAV clinical trials, there have been limited reports of pre-clinical studies evaluating the effectiveness of prednisone in dampening immune responses to AAV capsid. Prophylactic oral prednisone was evaluated in rhesus macaques receiving an IM injection of an AAVrh74-MCK-*GALGT2* vector.[81] Analysis of injected skeletal muscle revealed a reduction in infiltrating mononuclear cellular infiltrates, which consisted primarily of $CD8^+$ and $FoxP3^+$ regulatory T cells,[81] similar to what has been reported in human clinical trials investigating IM AAV gene delivery.[82,83] Infiltrating $CD8^+$ T cells displayed an exhausted phenotype with up-regulation of the death receptor PD1 as similarly reported in a study of $CD8^+$ T cell responses to wild-type AAV8 natural infection in rhesus macaques.[31] There was a trend toward reduced anti-AAVrh74 antibodies and IFN-γ ELISpot counts against capsid and GALGT2 epitopes in prednisone treated macaques however, due to the small group size did not reach significance. Elevated PDL2 expression on AAV transduced skeletal myofibers along with infiltrating regulatory T cells was suggested as the reason for persistent transgene expression in the presence of infiltrating $CD8^+$ T cells.

Transgene Product Immune Responses

Animal studies have identified multiple factors influencing transgene product immunity including AAV capsid and genome,[16,73,77,84,85] delivery route,[86,87] underlying mutation,[86] use of tissue-restricted promoters,[88] genetic background,[88] and disease-related inflammation.[89] Two examples from human clinical trials highlight this complexity. Adaptive $CD8^+$ T cell immune response against transgene product was identified in several Duchenne muscular dystrophy (DMD) patients treated with IM delivery of an AAV1 vector expressing the mini-dystrophin protein.[90] However, this response was not uniform among patients within the same dose cohort. In another clinical study, AAV1-hA1AT muscle gene transfer identified a cellular immune response against the transgene product, in a patient with a homozygous polymorphism in AAT combined with a rare HLA-C allele.[91] While not exhaustive, these examples show that the integration of many factors is involved in the acceptance or rejection of a therapeutic transgene product by the immune system

Humoral and Cell-Mediated Transgene Product Immunity

Muscle Gene Delivery

IM gene delivery of AAV vectors has been studied for both correction of muscle related diseases such as muscular dystrophies and Pompe disease and as a minimally invasive tissue for AAV transduction to produce FIX in hemophilia B and alpha-1 antitrypsin (AAT). However, IM delivery of AAV vectors often results in potent humoral and cell-mediated immune responses directed against the transgene product.[86,92,93] Early studies in mice showed that IM injection of AAV vectors induced functionally impaired $CD8^+$ T cells.[94,95] In wild-type C57BL/6 mice, the risk of a FIX antibody response was dependent on the dose and route of vector administration with IM delivery shown to be substantially more immunogenic compared to intraportal vector administration.[59] In hemophilia B dogs, the capsid serotype and transgene product expression levels were shown to impact the risk for FIX antibodies as IM delivery of an AAV2 vector was tolerated,[96] whereas treatment with an AAV1 vector resulted in the formation of anti-FIX antibodies.[92]

Studies in animals have also revealed that the genetic mutation plays an important factor in determining transgene product immunity.[97] Several lines of hemophilia B mice transgenic for different human *F9* mutations were generated[98] and backcrossed onto the C3H/HeJ background. Animals transgenic for a missense mutation of *hF9* expressing a non-functional FIX protein developed a weaker FIX immune response compared to null and early stop mutants following IM injection of an AAV2-*F9* vector.[86] Similar outcomes were observed in hemophilia B dogs with either a missense or null mutation for *canine F9*.[99] However, IM delivery of an AAV-*cF9* vector into hemophilia B dogs with a missense mutation showed a dose per injection site risk for a T cell-dependent anti-cFIX immunoglobulin G (IgG) response, suggesting that local inflammation may contribute to transgene immunity.[100] This is reflected in canine DMD models where disease-associated muscle inflammation requires the use of immune suppression for sustained expression of canine micro-dsytrophin.[101]

Transgene product acquired from transduced muscle cells is cross-presented on APCs[102] where TLR9 sensing of the AAV genome, MyD88, and IFNR were shown to be critical for productive transgene product-specific adaptive immune responses following IM AAV delivery.[11,19,84] An immunogenic hybrid capsid, rh32.33, was shown to provide modulatory signaling in activating transgene product immunity;[103] however, this activity was dependent on TLR9 activation, as depletion of TLR9 stimulatory CpG motifs in the AAV genome ablated transgene product immunity.[84] Further, TLR9 activation was shown to enhance transgene product immunity following AAV1-*hF9* IM delivery in C57BL/6 mice, which are normally tolerant to IM AAV1-hF9 gene transfer.[17] scAAV vectors, independent of

serotype, have shown enhanced transgene product humoral and cell-mediated immune responses in mice.[85] However, a *F9* missense mutation superseded the enhanced TLR9 activation of the scAAV1-*F9* vector, resulting in ablation of FIX $CD8^+$ T cell immunity.[104]

Several approaches have been tested in mice to limit transgene product immunity following IM AAV gene delivery including vector genome engineering to remove TLR9 stimulatory CpGs,[84] tissue-specific promoters and hematopoietic lineage microRNA (miRNA) targets 142-3p,[105] transient immune modulation,[40,106] and dual expression of transgene in liver and muscle.[107–110] Studies in large animal models have shown that the delivery route, direct IM injection versus regional vascular delivery, also impacts transgene immunity.[111] A recently published study reports 5-year inducible expression of the immunogenic doxycycline Tet-On system in NHPs following local regional delivery of an AAV vector with no detectable transgene product immune responses.[112]

Ocular Gene Delivery

Early preclinical studies for ocular gene therapy did not predict the inflammation observed in several patients receiving subretinal injection of an AAV2 vector.[113] However, lack of an immune response for ocular gene transfer in pre-clinical studies was expected, since the ocular compartment is considered to be immune privileged. One potential explanation is that the capsid serotype and delivery routes investigated may have contributed to masking this response. For example, RPE65-deficient dogs treated with subretinal injection of an AAV4-RPE65 vector did not report any incidence of ocular inflammation.[114] An AAV2-RPE65 subretinal injection into RPE65 null dogs demonstrated mild and transient ocular inflammation when using a highly purified vector prep,[115] whereas other studies in dogs reported much more severe inflammation.[116,117] More recent studies in NHPs show that subretinal delivery of an AAV8 vector can induce a transient local innate and adaptive immune response including recruitment of $CD8^+$ T cells and $CD20^+$ B cells into the retina.[21]

Liver Gene Delivery, Transgene Product Immunity, and Tolerance

Transgene product tolerance with AAV-targeted liver gene therapy is dependent on the induction and persistence of regulatory T cells (Tregs).[88,118–121] Supporting studies in a large animal model demonstrated that treatment of NHPs with daclizumab (anti-CD25 antibody), sirolimus, and mycophenolate mofetil broke tolerance against FIX protein following AAV2-F9 gene transfer due to depletion of Tregs.[122] Several studies in mice have shown that AAV liver transgene product tolerance is also dependent on protein expression levels.[87,88,123,124] The vector dose dependency of transgene product tolerance was further shown using an AAV8-EF1a-ova vector in C57BL/6 mice.[124] Mice receiving an intermediate vector dose expanded ova-specific $CD8^+$ T cells with an initial exhausted phenotype resulting in persistent transgene expression. However, over time, the ova-specific $CD8^+$ T cells became activated and eliminated ova-expressing hepatocytes,[124] whereas mice receiving the highest vector dose maintained stable ova expression with an undetectable ova-specific $CD8^+$ T cell response.[124] Although this study expressed ova from an ubiquitous promoter, tolerance induction was shown to be more effective when transgene product expression is restricted to hepatocytes by both promoter selection[88] and hepatotropic capsids.[87]

Most studies evaluating liver transgene product tolerance focused on secreted proteins, which could easily be measured in blood. Thus, it was unclear whether a non-secreted protein would promote immune tolerance. Liver transduction by an AAV2-LacZ vector prior to an Ad-LacZ vector resulted in sustained expression and suppression of transgene-specific $CD8^+$ T cell activation that was seen in Ad-LacZ only treated controls.[107] Expression of a membrane-bound ova protein in hepatocytes was shown to prevent airway-induced allergy mediated by Treg induction.[125] And finally, administration of an AAV8-*MOG* vector was shown to prevent and reverse disease in experimental autoimmune encephalomyelitis (EAE) mice, a model for multiple sclerosis.[126] Overall, the data show that tolerance is not dependent on a secreted transgene product.

Mechanistic studies in mice have identified an important role for transforming growth factor β (TGF-β) and IL-10,[127] glucocorticoid-induced TNFR-related protein (GITR) and GITRL interaction,[128] anergy, and deletion by Fas-FasL induced apoptosis.[88,119,129] Studies in immune-deficient mice transgenic for the ova $CD4^+$ TCR investigated where transgene presentation and conversion of $CD4^+$ T helper cells into Treg occurred following AAV8 liver gene transfer of a secreted or non-secreted ova antigen.[130] Mice expressing the non-secreted ova presented ova to $CD4^+$ T cells in the liver and liver draining lymph nodes and induced Treg were rapidly disseminated in the peripheral circulation,[130] whereas mice expressing a secreted ova had both extra-thymic and thymic Treg induction. Both macrophages and to a lesser extent cDCs were identified as critical APCs for $CD4^+$ T cell activation and peripheral Treg induction.[130]

AAV transgene product tolerance in the liver has allowed for transduction and supplemental gene expression using a more immunogenic vector[108] or tissue.[109,110,131] Further, several groups have demonstrated that established anti-drug antibodies to the transgene product could be eliminated by AAV liver gene transfer in murine[123] and canine[132,133] hemophilia models, as well as in a murine Pompe disease model.[134] Additionally, AAV liver transgene product tolerance was successful in preventing and reversing paralysis in the EAE mouse model.[126] There are several studies in large animal models supporting AAV-mediated transgene product tolerance. Liver-directed gene delivery of an AAV2 vector in hemophilia B dogs with a null mutation, which are prone to generate anti-FIX antibodies from IM delivery, provided over 10 years of FIX expression without any evidence of transgene product antibodies.[135,136] Another study in NHPs demonstrated that transgene tolerance could be induced with an AAV8-alpha-galactosidase A vector despite reduced transcription and expression of the transgene as compared to mice.[137]

It should be noted that AAV transgene product liver tolerance may not always be effective. Expression of human proteins such as

coagulation factors in NHPs can often induce a transgene antibody response and may require immune suppression to restore therapeutic expression.[53,54,138] A recent study evaluating a clinical AAV8 vector expressing human *UGUT1A1* in NHPs reported a mainly transgene product T cell response and found that peripheral transgene product T cell responses did not always correlate with the local T cell response in the liver.[139] Insulin gene therapy with an AAV8 vector in non-obese diabetic (NOD) mice found that immune suppression was needed to maintain insulin expression, suggesting that liver tolerance alone was not sufficient to overcome established autoimmunity.[140] However, it should be noted that NOD mice have a genetic predisposition for autoimmunity and that the target level of insulin required for therapeutic benefit may be below the threshold need for tolerance induction.

Conclusions

Despite a growing level of insight into AAV vector immunity from animal studies, it is unclear whether a single model will be able to completely predict human responses. Mouse models have provided data on feasibility of disease correction, potential risks of transgene product and vector immunity in the context of disease model, AAV serotype, target organ, and delivery route. Recent studies have also begun to identify critical mechanisms of AAV vector sensing and activation of adaptive immune responses and tested vector modifications and targeted therapies to blunt these responses. However, the genetic uniformity of inbred strains and absence of natural infection with AAVs make it difficult to fully extrapolate outcomes in human studies. Studies in larger animal systems including dogs and NHPs have also provided more valuable insights into vector-mediated safety and to some extent efficacy. The scarcity of genetic NHP disease models and high risk of transgene immunity when human proteins are expressed in dogs and NHPs make it difficult to take advantage of their longer lifespans to evaluate clinical vector preparations for long-term transgene product expression, efficacy, and vector and transgene product immunity.

Ultimately, humans will likely be the best animal model to study AAV immunity with experience gained from the use of approved AAV biologics and clinical trials. However, several important questions still remained unanswered. Can testing of peripheral blood mononuclear cells predict $CD8^+$ T cell responses against AAV capsid and rejection of therapy and how can we better screen patients? Is the immunotoxicity in humans a *de novo* immune response or a recall response? Are there toxicities unrelated to vector immunity? What is the best immune suppression protocol, agent, and treatment window to prevent capsid related immunotoxicity? What is the optimal vector dose for each capsid serotype to avoid immunity while providing therapeutic transgene expression? Continued research into AAV vector immunity and associated toxicities and the generation of new and improved animal models may help to address these questions.

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