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Research paper

# Preclinical models to assess the immunogenicity of AAV vectors

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ABSTRACT

Although gene transfer using adeno-associated virus (AAV) vectors has made tremendous progress in recent years, challenges remain due to vector-specific adaptive immune responses. Specifically, AAV-neutralizing antibodies reduce AAV-transduction rates, while $CD8^+$ T cells directed to AAV capsid antigens cause rejection of AAV-transduced cells. This has been addressed clinically by excluding humans with pre-existing AAV-neutralizing antibodies from gene transfer trials or by using immunosuppression or reduced doses of vectors expressing improved transgene products to blunt or circumvent destructive T cell responses. Although these approaches have met with success for treatment of some diseases, most notably hemophilia B, they may not be suitable for others. Pre-clinical models are thus needed to test alternative options to sidestep pre-existing AAV-neutralizing antibodies, to prevent their induction following gene transfer and to block the detrimental effects of $CD8^+$ T cells directed to AAV capsid antigens. This chapter describes some of the available, although not yet perfect, models that can assess immune responses to AAV gene transfer.

## 1. Introduction

Gene transfer can permanently correct single gene defects. AAV vectors due to their ease of construction, wide tissue tropism and lack of pathogenicity are favorite vehicles for long-term gene replacement therapy. Nevertheless, challenges due to reduced transduction in presence of AAV-neutralizing antibodies [1,2] or immunological rejection of transduced cells by $CD8^+$ T cells directed against AAV capsid antigens remain [3,4].

Transfer of low doses of AAV vectors to immunoprivileged sites, such as the subretinal space of the eye, has achieved clinical benefits without eliciting overt immune responses [5,6]. In contrast, initial systemic gene transfer trials with high doses of an AAV vector expressing human factor 9 (h.FIX) applied to the liver for treatment of hemophilia B resulted in increases in AAV capsid-specific $CD8^+$ T cell responses accompanied by loss of FIX expression [3]. Short-term courses of immunosuppressive drugs such as prednisolone [4] or lowering the amount of vector upon optimization of the therapeutic transgene product [7] can inhibit, circumvent, or prevent immune responses and thus allow for sustained h.FIX expression. With low vector doses, some AAV gene transfer recipients never develop a full-blown destructive immune response and continue expression of the transgene product without need for immunosuppressive drugs. In other individuals, frequencies of circulating AAV capsid-specific $CD8^+$ T cells increase accompanied in some but not all cases by evidence of tissue destruction and loss of the transgene product. Some of the latter group of individuals benefit from immunosuppression while others, despite immunosuppression, experience a reduction in transgene product expression [8]. Alternatively, AAV vectors can be given into muscles. Induction of cellular immune responses to AAV capsid were reported in one trial that used intramuscular AAV gene transfer [9], while another observed increases of transgene product-specific T cells [10]. A third trial reported stable transgene product expression without subjecting gene transfer recipients to immunosuppressive drugs, a finding that was linked to induction of immune-inhibitory $CD4^+$ regulatory T cells [11].

## 2. Why do we need pre-clinical animal models?

Additional pre-clinical research is needed to develop algorithms that will allow predicting if a given individual will tolerate the AAV vector or may reject it. It would be helpful to know when the critical phase, during which immune responses endanger the transduced cells, has passed so that close monitoring of the gene transfer recipient can be relaxed and if and when immunosuppression should be initiated and when it can be stopped. It would also be important to know if, in an individual is at risk for rejection, such an event can or cannot be controlled by immunosuppressive drugs. Outcome is likely affected by multiple parameters, such as peculiarities of the vectors including differences between serotypes, which affect vector tropism, speed of uncoating and intracellular trafficking patterns [12–14] or the vectors genome composition, which drives innate immune responses [15–17]. The transgene, route of application and vector dose will play roles, as

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will characteristics of each patient, such as age, gender, ethnicity, and genotype of major histocompatibility antigens, all of which affect immune responsiveness, or level of pre-existing immunity to AAV. Pre-clinical models are also needed to validate new approaches to circumvent AAV-neutralizing antibodies, to block their induction following AAV gene transfer and to prevent activation or expansion of AAV capsid- or transgene product-specific $CD8^+$ T cells.

## 3. Basic problems with pre-clinical animal models for AAV

One of the problems that plagues pre-clinical studies of AAV-mediated gene transfer is that animal models do not mirror treatment outcome in humans. The tropism of AAV vectors that likely affects induction and effector functions of AAV vector-induced T cell responses differs between experimental animals, such as mice, and humans, as was shown with immunodeficient human liver chimeric mice [18,19]. Mice, dogs or nonhuman primates do not reject AAV-transduced cells [20–23] even if they respond to AAV gene transfer with capsid- or transgene product-specific $CD8^+$ T cell responses [24]. Our initial interpretation was that humans due to childhood infections with AAV combined with a helper virus develop immunological memory to AAV capsid that once triggered by AAV gene transfer is responsible for elimination of vector-transduced cells [25]. Although this theory is compatible with lack of rejection of AAV vectors in mice or dogs, which do not naturally become infected with the AAV serotypes that are used for human gene transfer, it is hard to reconcile with lack of rejection in nonhuman primates, which carry AAVs that are closely related to those in humans [26,27]. Although pre-existing T cell responses in nonhuman primates are distinct from those in humans [27], which might contribute to discrepancies in AAV gene transfer outcome, other reasons need to be explored. In addition, although all evidence points towards AAV capsid-specific $CD8^+$ T cells as the culprits for loss of transgene product expression, it has not yet been confirmed if indeed rejection is linked to recall rather than to primary responses. In fact, kinetics of rejection that in general happens late at 4–10 weeks after gene transfer is more compatible with primary than memory responses, as the latter generally come up rapidly [28].

Pre-clinical animal models have been developed to study immune responses to AAV vectors. Here we discuss some of the most promising models emphasizing their worth and limitations.

## 4. Human liver mouse chimeras

Immunodeficient NOD *rag1−/− il2rgnull* mice with a genetic knock-out of the fumarylacetoacetate hydrolase gene, which encodes an enzyme needed for the hydrolysis of 4-fumarylacetoacetate, have been generated [29]. These mice develop liver necrosis unless they are maintained on nitisinone (NTBC). Upon withdrawal of NTCB the mice can be engrafted with fetal human hepatocytes, which expand within the mouse liver [30]. The human liver mouse chimeras have been used to assess AAV tropism to hepatocytes and shown that human hepatocytes are 20 times less sensitive to transduction with AAV8 vectors compared to mouse hepatocytes [18]. A follow-up study confirmed these results and reported that an engineered AAV3 capsid with two amino acid changes has high tropism for human hepatocytes while AAV5, 8, and 9 vectors are more efficient in transducing mouse hepatocytes [19]. Such studies are exceedingly valuable for pre-clinical assessment of vectors intended for hepatic transfer, as vector dose, which must be increased for vectors with low tropism for hepatocytes, is one of the most important drivers of induction of destructive immune responses [3].

Human liver mouse chimeras can further be transplanted with hematopoietic stem cells from matched fetal donors [30]. These cells will differentiate into cells of the innate and adoptive immune system, which would in theory generate a mouse that can be used to test for AAV vector immunogenicity within the context of a partially human liver and a human immune system. Although hypothetically very attractive, this model has not yet been fully developed and still faces challenges, such as difficulties in procurement of matching fetal liver and hematopoietic stem cell samples, high variability of human chimerism and short lifespan of the chimeric mice [30]. Immune responses in the chimeras against a viral pathogen have been achieved but they were very low [31], which may reflect that crucial structures such as lymphoid tissues are missing in the reconstituted NOD *rag1−/− il2rgnull* mice, and that vessels and tissue-derived inflammatory signals are of mouse origin.

## 5. Adoptive transfer of AAV capsid-specific $CD8^+$ T cells into AAV vector-injected recipient mice

My laboratory developed a very sensitive method to assess $CD8^+$ T cell responses to AAV capsid in inbred mice [32]. Recipient mice are injected intravenously (i.v.) with an AAV vector. Donor mice, which are congenic for Thy1 or another marker expressed on $CD8^+$ T cells to allow for their tracking within the recipients, are immunized with an adenovirus vector or another type of vaccine expressing AAV capsid. Once the AAV capsid-specific $CD8^+$ T cells transition towards a more resting stage, donor splenocytes are isolated, stained with carboxyfluorescein succinimidyl ester (CFSE) or another intracellular dye that upon cell division is equally distributed into the daughter cells. The labeled cells are then transferred i.v. into the recipient mice. Lymphocytes are isolated from recipient mice 10 days after cell transfer, stained for live cells, the congenic Thy1 marker of the donor mice and CD8. AAV capsid-specific $CD8^+$ T cells are identified by a specific MHC class I tetramer or by intracellular cytokine staining upon a brief *in vitro* stimulation with peptides for the immunodominant epitopes within the AAV capsid. Cells are then analyzed by flow cytometry and blots are gated onto live AAV capsid-specific $CD8^+$ donor cells. Loss of CFSE within those cells serves as a read-out for proliferation. Proliferation of donor $CD8^+$ T cells in turn is driven by the epitopes within the AAV capsid present within the recipients. Recipients that did not receive an AAV vector to determine level of background proliferation of donor $CD8^+$ T cells that occurs independent of their cognate antigen control the assay. The method can be rendered more sensitive by incorporating several copies of SIINFEKL, a peptide that presents the immunodominant $CD8^+$ T cell epitope of ovalbumin, into capsid protein VP2 of AAV. Incorporation of a foreign epitope also allows for testing of AAV vectors, such as AAV8, that fail to induce capsid-specific $CD8^+$ T cell responses in mice [33]. To assess a proliferative response of memory $CD8^+$ T cells donor mice are immunized against SIINFEKL prior to their transfer. Transfer of CFSE-labeled splenocytes from OT-1 mice, which carry the transgenic $CD8^+$ T cell receptor for SIINFEKL, can test for primary proliferative T cell responses.

This method is not suited to assess parameters that drive the initial stimulation of AAV capsid-specific memory $CD8^+$ T cells. It could be used to assess induction of primary cells in the OT-1 cell transfer system with the caveat that SIINFEKL is an epitope with unusually high avidity to its restricting element. The model is not suited to study the effector phase as despite transfer of capsid-specific memory $CD8^+$ T cells or naïve OT-1 T cells into recipients of SIINFEKL-modified AAV vectors, mice fail to show evidence of tissue damage (unpublished) and maintain stable levels of the transgene product. The transfer experiments are designed to define parameters that affect T cell proliferation and as such can be used to determine kinetics of the display of antigenic AAV capsid epitopes. Using this method, we showed that a proliferative memory $CD8^+$ T cell response to AAV2 capsid can be seen for up to 3 weeks after gene transfer while proliferation of naïve SIINFEKL-specific $CD8^+$ T cells into mice injected with a SIINFEKL-modified AAV2 capsid remains detectable longer for up to 10 weeks [32]. AAV8 capsid seems to persist even longer and still triggers a response of OT-1 $CD8^+$ T cells to SIINFEKL incorporated into AAV8's capsid at 6 months after transfer [33].

Roland Herzog modified this method by transferring *in vivo* activated and then *in vitro* expanded AAV capsid-specific $CD8^+$ T cells into AAV2-hF.IX vector-bearing T- and B-cell deficient C.129S7(B6)-Rag1tm1Mom/J recipients, which shortly before and after cell transfer were treated with lipopolysaccharide (LPS) for further immune activation [34]. Mice developed increases in transaminases and hF.IX levels were reduced in recipients of AAV capsid-specific $CD8^+$ T cells. An AAV2 capsid mutant vector, in which surface exposed tyrosine residues were exchanged with phenylalanine to reduce the vector's proteosomal degradation and thereby access of the capsid's epitopes to MHC class I molecules within the endosomal reticulum [35], was targeted less efficiently by the transferred T cells. This method again is unsuited to assess induction of capsid-specific $CD8^+$ T cells but it can serve to assess parameters that influence the effector phase [34].

## 6. Immunogenic AAV vectors

AAV vectors used for human gene transfer tend to be poorly immunogenic in animals. Some serotype like AAV2 induce in mice low capsid-specific $CD8^+$ T cell responses while others such as AAV8 do not [36]. As epitopes are shared between these serotypes [37,38], this likely reflects differences in the vectors' receptor usage and in their ability to efficiently transduce mouse dendritic cells. One hybrid AAV vector, termed AAVrh32.33, which was isolated from rhesus macaques [39], was found to induce potent transgene product- and capsid-specific $CD8^+$ T cell responses in mice that resulted in rejection of vector-transduced cells within a few weeks [40]. Studies in nonhuman primates confirmed that an AAVrh32.33 vector expressing antigens of HIV-1 stimulated high frequencies of transgene product-specific immune responses that could be boosted with a heterologous vaccine platform [39]. This differs from immune responses to other AAV vectors, which in mice were shown to induce defective transgene product-specific T cell responses that failed to expand after re-exposure to their cognate antigen [41,42]. An AAVrh32.33 vector expressing the highly immunogenic beta-galactosidase (β-Gal) as its transgene product was then used to assess the role of CpG motives. These are pathogen-associated molecular patterns (PAMPs) that upon interaction with TLR9 trigger innate immune responses, which in turn drive the adaptive immune responses that cause rejection of transduced cells [43]. Results showed that a CpG-depleted AAVrh32.33 vector given to muscle failed to induce capsid- or transgene product-specific T cell responses in mice, which allowed for persistent expression of the vector's transgene [44]. This model may be very useful to assess characteristics of destructive AAV capsid-specific T cell responses in pre-clinical models but some caveats should be pointed out. Studies thus far were conducted with highly immunogenic transgene products, such as β-Gal, a bacterial protein, green fluorescent protein (GFP), which is found in marine organisms, firefly luciferase or antigens from HIV-1 or influenza virus. It is thus not clear if and to what degree the T cell response to AAVrh32.33 capsid contributed to the rejection of transduced cells or if this was mainly driven by the more potent responses to the transgene products. In non-human primates, some of which should mirror the human immune status to clinically used AAV vectors by having pre-existing T cells to AAVrh32.33, responses to its capsid were not tested for [39]. It is thus not clear if such T cell responses were elicited. Furthermore, as was shown in mice, depletion of CpG motifs from the AAVrh32.33 vector, but for those essential for the integrity of the inverted terminal repeats, blocked induction of transgene or capsid-specific T cell responses [44] unlike in humans, where in a trial with a CpG-reduced double-stranded AAV8 vector for F.IX some of the gene transfer recipients showed elevations in transaminase and developed increased frequencies of AAV capsid-specific T cells, which triggered administration of immunosuppressive drugs [4]. In humans, CpG-depletion of the AAV8 vectors did thus not prevent capsid-specific T cell responses and initiation of rejection. This discrepancy could reflect that in humans PAMPs, other than CpGs, within the AAV vectors may play a role in driving adaptive immune responses [45]. Alternatively, it may reflect that experiments in mice, including those with AAV8 vectors carrying a double stranded genome [46], targeted an immune system that was naïve to AAV while human AAV recipients have immunological memory. CpG motives within AAV vectors may be essential to induce primary $CD8^+$ T cell responses by driving maturation of plasmacytoid and conventional dendritic cells [9], but they may not be needed to elicit recall responses, which are less dependent on antigen presentation by mature dendritic cells. To add complexity, it is also feasible that once a recall response to AAV capsid is initiated the ensuing cytokine response may circumvent the need for CpG-driven innate responses to promote activation of naive T cells to hitherto unencountered epitopes of the vector. All of this can and should be tested to validate the usefulness of AAVrh32.33 as a pre-clinical tool to study immune responses to the capsid of AAV vectors.

In theory insertion of a potent $CD8^+$ T cell epitope such as SIINFEKL into the capsid of AAV vectors should render such vectors more immunogenic. Indeed, Jude Samulski's group reported that an AAV2 vector, carrying SIINFEKL within its capsid, elicited a SIINFEKL-specific $CD8^+$ T cells response in mice, which could be enhanced when mice were immunized with SIINFEKL peptide-pulsed dendritic cells prior to vector transfer [47]. The SIINFEKL-specific $CD8^+$ T cells were lytic, caused a modest rise in transaminases upon hepatic AAV vector transfer and attenuated transgene product expression. The authors concluded that such vectors would be suited to determine response kinetics to AAV vectors and assess strategies to block responses. Again, there are some caveats that need to be pointed out. We used AAV2 vectors expressing SIINFEKL within their capsid but these vectors did not induce a SIINFEKL-specific $CD8^+$ T cell response and expression of the transgene product was sustained and comparable in magnitude to that achieved with AAV2 vectors with a wild-type capsid [32]. Discrepancies in results could potentially reflect differences in transgenes; Jude Samulski's group used luciferase or alpha1 anti-trypsin while our experiments were mainly conducted with vectors expressing hF.IX. In the Samulski study [47], mice were rendered immune to SIINFEKL by repeated injections of SIINFEKL peptide-pulsed dendritic cells; they were injected 10 days later with the modified AAV2 vector. The vectors were thus given at the height of the SIINFEKL-specific $CD8^+$ T cell effector cell response and accordingly transgene product expression was attenuated from the onset. This is markedly different from the human immune status to AAV capsid and their reaction to AAV gene transfer. Humans have low frequencies of circulating AAV capsid-specific $CD8^+$ T cells, which are too low for detection by ELISpot assays but can be identified by multicolor flow cytometry in about 50% [27] and by more sensitive methods such as Tetramer-Associated Magnetic Enrichment in 100% of human adults [48]. Most of the circulating AAV capsid-specific $CD8^+$ T cells exhibit a phenotype typical for resting memory $CD8^+$ T cells. Effector $CD8^+$ T cells can only be detected at low frequencies in a fraction of human adults [27]. Accordingly, upon AAV gene transfer transgene product expressing initially rises while capsid-specific T cell responses increase with a delay reflecting either recall of pre-existing memory cells or *de novo* induction of naïve cells either of which may then cause a reduction in levels of AAV transduced cells [49]. The response in humans is thus not mediated by pre-existing effector $CD8^+$ T cells but rather by cells that require activation followed by expansion. Additional experiments are needed with this model to test if a recalled SIINFEKL-specific memory $CD8^+$ T cell response or a *de novo* response would result in rejection of vector-transduced cells with kinetics like those observe in human AAV vector recipients.

## 7. *In vitro* systems

Effector functions of AAV capsid-specific $CD8^+$ T cells can be studied *in vitro*. T cells can be isolated from blood of healthy human adults and those that are specific for AAV capsid can be expanded *in vitro* by repeated stimulation with AAV particles, peptide pools of AAV capsid

antigens or individual peptides carrying define epitopes of AAV capsid.

Human CD8$^+$ T cells can be used to identify epitopes within AAV capsid [50]. They can be used to determine if certain types of cells such as hepatocytes become susceptible for lysis upon AAV transduction and how lysis can be inhibited [51]. Human T cell lines are not suited to assess the kinetics of responses to AAV capsid, which are likely driven by the speed of AAV uncoating and intracellular degradation of capsid antigens, as primary cells which would faithfully mimic such processes, are generally short-lived.

## 8. AAV capsid-specific antibodies

Humans commonly carry neutralizing antibodies to AAV induced upon natural infections [52]. Such antibodies even at low titers strongly reduce AAV transduction rates upon systemic application of AAV vectors [53]. AAV gene transfer to peripheral sites triggers an AAV capsid-specific B cell response that gives rise to AAV-neutralizing antibodies [54,55], which in turn preclude re-administration of the same or a serologically related AAV vector. Studies in experimental animals showed that effects of AAV-specific neutralizing antibodies can be circumvented by using distinct AAV serotypes for sequential gene transfer [10] or by genetic capsid modifications that deplete binding sites for neutralizing antibodies without affecting AAV vector yields or the vector's tropism or transduction rates [56,57]. Induction of AAV-specific antibodies can be prevented by B cell depletion using antibodies to the pan B-cell marker CD20 (Rituximab) thus allowing for vector readministration [58]. The impact of AAV-specific antibodies on AAV gene transfer and avenues to circumvent such antibodies or to reduce their induction can readily be studied in pre-clinical animal models. For example, studies in mice and nonhuman primates showed that AAV-neutralizing antibodies block liver transduction upon i.v. injection of a matching AAV vector but allow for efficient local transduction upon intramuscular injection [59]. Another study showed again in mice and nonhuman primates that so-called empties, which are AAV vectors, which failed to encapsidate a genome, can serve as decoys to reduce the impact of AAV neutralizing antibodies on hepatic AAV transduction [60].

Methods to test AAV capsid-specific antibody responses to natural AAV infections or AAV gene transfer have limitations. Antibody assays are not standardized and it is thus hard to evaluate if results obtained by one laboratory can be repeated by others. Assays for titration of AAV-specific antibodies can be conducted *in vitro* using cell-based neutralization assay with vectors expressing reporter genes [61] or by assessing antibody binding in ELISAs, which can give insight into antibody isotypes but provide limited information on antibody functions [62]. Alternatively, neutralizing antibodies can be tested in mouse transfer experiments, which measures reduction in AAV transduction rates in presence of passively transferred sera [62]. Although the *in vivo* neutralization assay is more sensitive and probably more relevant [63] than *in vitro* assays [64], it is unfortunately very cumbersome especially if used to assess titers rather than presence vs. absence of neutralizing antibodies.

## 9. Summary

Preclinical models for AAV mediated gene transfer are available but still have limitations. *In vitro* systems can identify parameters that influence the effector phase of AAV capsid-specific CD8$^+$ T cells but they give no insight into conditions that drive their initial activation and expansion. Highly immunogenic AAV vectors based on AAVrh32.33 can be used to assess both induction and effector phases of AAV capsid-specific CD8$^+$ T cells but are not suited to determine if, how and to what degree differences in the biology of different AAV serotypes influence CD8$^+$ T cell responses. Adoptive transfer models give no insight into the initial stimulation or recall of AAV capsid-specific CD8$^+$ T cells but they are useful to measure responses kinetics to different types of vectors and to some degree evaluate interventions that interfere with expansion or effector functions of AAV capsid-specific CD8$^+$ T cells.

## Conflict of interest

HC Ertl has sponsored research agreements with Spark Therapeutics and Pfizer.

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