Rev. Med. Virol. 2013; **23**: 399–413.
Published online 10 September 2013 in Wiley Online Library
(wileyonlinelibrary.com)
DOI: 10.1002/rmv.1762

Reviews in Medical Virology

REVIEW

Adeno-associated virus (AAV) vectors in gene therapy: immune challenges and strategies to circumvent them

Sangeetha Hareendran[1], Balaji Balakrishnan[2], Dwaipayan Sen[2], Sanjay Kumar[1], Alok Srivastava[1,2] and Giridhara R. Jayandharan[1,2]*

[1]*Centre for Stem Cell Research, Christian Medical College, Vellore, Tamil Nadu, India*
[2]*Department of Hematology, Christian Medical College, Vellore, Tamil Nadu, India*

SUMMARY

AAV-based gene transfer protocols have shown remarkable success when directed to immune-privileged sites such as for retinal disorders like Lebers congenital amaurosis. In contrast, AAV-mediated gene transfer into liver or muscle tissue for diseases such as hemophilia B, α1 anti-trypsin deficiency and muscular dystrophy has demonstrated a decline in gene transfer efficacy over time. It is now known that in humans, AAV triggers specific pathways that recruit immune sensors. These factors initiate an immediate reaction against either the viral capsid or the vector encoded protein as part of innate immune response or to produce a more specific adaptive response that generates immunological memory. The vector-transduced cells are then rapidly destroyed due to this immune activation. However, unlike other viral vectors, AAV is not immunogenic in murine models. Its immunogenicity becomes apparent only in large animal models and human subjects. Moreover, humans are natural hosts to AAV and exhibit a high seroprevalence against AAV vectors. This limits the widespread application of AAV vectors into patients with pre-existing neutralising antibodies or memory T cells. To address these issues, various strategies are being tested. Alternate serotype vectors (AAV1-10), efficient expression cassettes, specific tissue targeting, immune-suppression and engineered capsid variants are some approaches proposed to minimise this immune stimulation. In this review, we have summarised the nature of the immune response documented against AAV in various pre-clinical and clinical settings and have further discussed the strategies to evade them. Copyright © 2013 John Wiley & Sons, Ltd.

Received: 4 June 2013; Revised: 8 August 2013; Accepted: 9 August 2013

INTRODUCTION

Clinical application of a viral vector for gene therapy requires that sustained therapeutic levels of the transgene are achieved, with no apparent vector-related toxicities in the patient. The commonly used agents for gene correction or gene replacement include recombinant vectors based on retrovirus, lentivirus, adenovirus or AAV [1–3]. Of these, adeno-associated virus-based vectors have gained prominence in gene transfer studies due to their non-pathogenic nature and low immunogenicity compared to other viral vectors such as adenovirus [4]. AAV is a small, non-enveloped virus of ~22 nm in size, belonging to *Parvoviridae* family and *Dependovirus* genus. It has a single stranded (ss) DNA genome of ~4.7 kb which contains two open reading frames encoding the *rep* and *cap* genes flanked by 145 base pair long ITR sequence. Productive infection of the virus requires the presence of other helper viruses such as adenovirus or herpes simplex virus [5]. During the production of rAAV vectors, the transgene cassette is incorporated between the ITR containing plasmid whereas the rep-cap is supplied *in trans* along with the helper function genes in a triple plasmid transfection protocol [6]. This generates replication defective vectors which exist as episomes in host cells. Currently,

*Correspondence to: G. R. Jayandharan, Department of Hematology and Centre for Stem Cell Research, Christian Medical College, Vellore-632004, Tamil Nadu, India.
E-mail: jay@cmcvellore.ac.in

Abbreviations used
AAV, adeno-associated virus; APC, antigen-presenting cells; DC, dendritic cells; DMD, Duchenne muscular dystrophy; FIX, coagulation factor IX; HSPG, heparin sulphate proteoglycan; ITR, inverted terminal repeat; KC, Kupffer cells; LPL, lipoprotein lipase; MIP-2, macrophage inflammatory protein 2; Nab, neutralising antibodies; NF-κB, nuclear factor-kappa B; PAMPs, pathogen-associated molecular patterns; PBMNCs, peripheral mononuclear cells; PRR, pattern recognition receptors; rAAV, recombinant adeno-associated virus; sc, self-complementary; TLR, Toll-like receptor; Tregs, regulatory T cells; UPR, unfolded protein response; UPREs, UPR elements.

12 different AAV serotypes (AAV1-12) have been utilised as gene therapy vectors while several other variants are also known to exist [1].

In pre-clinical studies, AAV vectors have shown an ability to transduce a wide variety of tissues, provide stable transgene expression and exhibit relatively low immunogenicity [1]. AAV serotype 2 is the prototype vector and has been extensively tested for its safety in pre-clinical and clinical studies [7]. Other alternate serotypes [AAV1, AAV5, AAV8 and AAV9] have also generated promising pre-clinical data for treatment of a variety of disease states such as muscular dystrophy, hemophilia and α1- anti trypsin deficiency [7–9]. These vectors, when compared to AAV2, show remarkably diverse tissue tropism and low immune activation [8,10].

In human clinical trials, AAV vectors have shown promise for gene delivery into post-mitotic tissues such as liver, retina and brain [1]. Indeed, AAV8 vectors were successfully evaluated in a liver-directed clinical trial for hemophilia B where patients have now shown multi-year therapeutic benefit [11]. However, host and vector-related immune challenges not predicted previously in animal models remain. In a phase I/II dose escalation clinical trial in patients with severe hemophilia B, liver-directed infusion of AAV2 resulted in therapeutic expression of FIX transiently for ~6 weeks [12]. Later, analysis of PBMNCs collected from a patient in the high dose group revealed a capsid-specific CD8+ T-cell response that destroyed vector-transduced hepatocytes [13]. Similar responses have been noted in multiple other trials as well [14,15] including in patients treated with AAV1 vectors for LPL deficiency [16] and AAV2 vectors for DMD [9].

The immunological sequel that emerged in clinical trials using AAV vectors advocates a better understanding of the vector immunology. This knowledge will be a prerequisite in designing optimal gene transfer strategies. Our review thus attempts to summarise the data available from literature on the nature and type of immune response directed against AAV vectors and further discuss strategies which could be used to circumvent them.

NATURE OF IMMUNE RESPONSE DIRECTED AGAINST AAV

Innate immunity

The immunogenic profile of AAV was considered relatively low owing to its poor transduction of APC such as macrophages and mature DC [17]. However, APCs can take up exogenous AAV antigenic peptides by endocytosis and display them on MHC Class I molecules by a process termed cross presentation. This mechanism of antigen presentation is believed to trigger CD8+ T-cell response against the AAV-encoded transgene [18]. However, immature DCs can be infected by AAV vectors *ex vivo* and these cells when adoptively transferred into immune-competent mice result in T-cell-mediated elimination of transduced muscle fibres in a CD40-ligand-dependent manner [19]. Nonetheless, direct intramuscular administration of the vectors did not cause loss of transgene expression as it failed to recruit enough immature DCs to the site of AAV injection [20]. These data suggest that AAV infection of immature APCs may be the trigger for an innate immune response.

The innate immune system also includes the complement system which has been known to be an essential element of the host anti-viral response [21]. Immuno-precipitation studies have validated that AAV capsid directly binds and interacts with the C3 complement proteins, which can lead to vector opsonisation and macrophage activation. Regardless of poor transduction and lack of measurable transgene expression, AAV2 vector internalisation was found to be enhanced in primary mouse bone marrow macrophages and differentiated THP-1, human acute monocytic leukemia cell line in the presence of normal serum. This coincided with the activation of these cells, as ascertained by the expression of various cytokines and chemokines such as MIP-2, IL-1, IL-8 and MIP-1. *In vivo* experiments using complement receptor 1/2- and C3-deficient mice underscored the additional role of complement in the generation of Nab against AAV2 [21]. Figure 1 gives an overview of the role of complement system and other innate immune mechanisms noted against AAV following their internalisation into the cell.

The innate immune system actively participates in shaping adaptive response, mostly by the engagement of PRR which recognise PAMPs [26]. This interaction triggers signalling pathways that lead to the production of pro-inflammatory cytokines and the recruitment of infiltrating macrophages and neutrophils. These cells can directly eliminate the target microbe or initiate a more specific T-cell and B-cell response. AAV2 is known to activate plasmacytoid DCs *via* the TLR signal

DOI: 10.1002/rmv

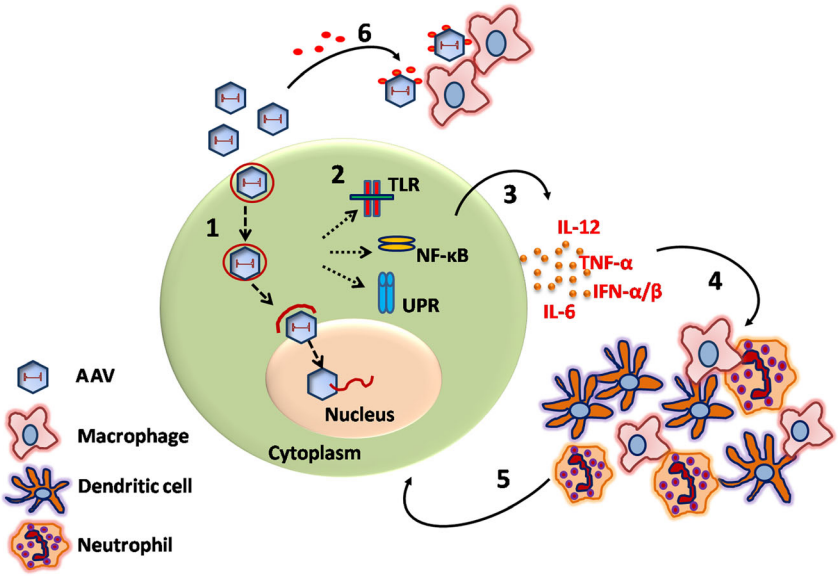

Figure 1. Innate immune response against AAV. (1) AAV enters into the cell by receptor-mediated endocytosis, travels through the cytosol and is released following the acidification in the endosomes. After its endosomal escape, it enters the nucleus, wherein uncoating of the viral capsid takes place followed by the release of the AAV genome and induction of gene expression [22]. (2) Depending on the target cell and appropriate cues, internalisation of AAV may trigger the activation of various innate immune signalling pathways such as TLR, NF-κB and UPR [23–25] which leads to the secretion of pro-inflammatory cytokines and chemokines as shown in 3. (4 and 5) These molecules further activate and cause the infiltration of innate immune cells like neutrophils, DCs and macrophages to the transduced cell thereby eliminating it. (6) AAV vectors can also be opsonised by binding of complement proteins, e.g. C3, which can cause the activation and endocytosis by macrophages [18]

transduction (TLR9-MyD88) pathway to produce type I IFN [27]. TLR9 signalling was found to be critical for activation of CD8+ T-cell response against transgene or AAV capsid, and also generation of Nab, as demonstrated in studies using *TLR9* or *MyD88*-deficient mice. The induction of this endosomal PRR pathway was independent of the transgene or AAV serotype [27]. Another study reported that hepatic gene transfer of scAAV vectors in mice leads to dose-dependent increase in innate responses via the TLR-9 signalling [23]. These data suggest the important role played by TLR pathways in activating the innate immune response against AAV vectors.

Activation of transcription factors associated with immune reactions to AAV has also been described [28]. NF-κB, which is a central regulator of inflammatory response, is known to be involved in this process. AAV infection of human cells activated the alternate NF-κB pathway [24]. Liver-directed administration of AAV in mice showed activation of the canonical pathway by 2 h and sequential activation of the alternate pathway by 9 h in liver tissue. The transient inflammatory cytokine response and anti-AAV antibody production mediated by NF-κB upregulation was effectively blocked with Bay 11, a pharmacological NF-κB inhibitor, distinctly highlighting the role of NF-κB in modulating immune response to AAV [24].

We have recently shown that AAV vectors activate distinct arms of the cellular UPR signalling cascade [25]. Available evidence suggests that AAV intracellular trafficking occurs by retrograde transport [29,30](Figure 2). During this process, we reasoned that a massive influx of the AAV particles into the ER may activate the pathway as an ER stress response mechanism. The UPR is a cytoprotective mechanism elicited to maintain cellular homeostasis (Figure 3)[31,32]. This process decides the ultimate fate of the cells under stress, committing them to either undergo apoptosis or upregulate cytoprotective mechanisms. Such protective mechanisms include UPR-mediated activation of innate immune response [33]. Several reports suggest that the ER stress activates NF-κB pathway through IRE1α, which then translocates to the nucleus leading to increased expression of pro-inflammatory genes (Figure 3)[34,35]. In our studies [25], we observed that scAAV genome triggered a more prominent UPR, the PERK and IRE1

DOI: 10.1002/rmv

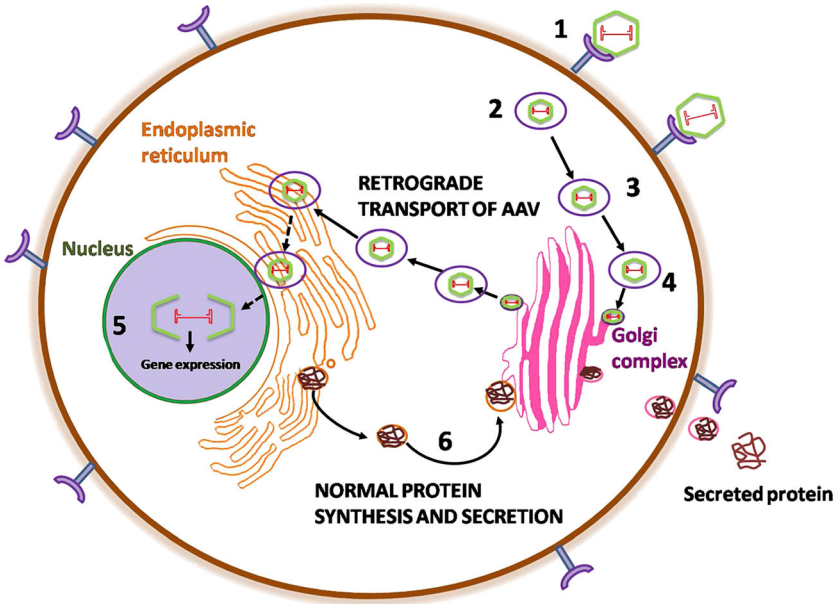

Figure 2. Retrograde transport of AAV and endoplasmic reticulum stress. (1) AAV binds to its cell surface receptor/co-receptor and forms a complex [36]. (2) AAV–receptor complex is internalised and travels in the cytosol via endosomal vesicles. (3) Early endosome to late endosome transition of AAV eventually starts the retrograde transport of AAV [29]. (4) The endosomal vesicles fuse to the Golgi complex from where the AAV pass to accumulate in the ER. (5) The virus then enters the nucleus, uncoats and maintains the genome in an episomal form [30]. (6) Normal protein synthesis involving protein processing in the ER and its transport through Golgi complex is also highlighted [37]

pathways in particular, than ssAAV. In addition, pharmacological inhibition of UPR suppressed the NF-κB-mediated anti-viral innate immune response directed against scAAV2 vectors while modestly improving the transgene expression in murine liver [25]. Taken together, these data underscore the role played by various innate arms of the immune system in initiating an inflammatory reaction against AAV and leading to either vector clearance or activation of the more specific adaptive response.

Adaptive immunity

Pre-existing immunity. Multiple species are known to serve as hosts to AAV infection [40,41]. The natural exposure to various AAV leads to the generation of anti-AAV antibodies in the sera. Boutin *et al*. documented the prevalence of total IgG antibodies against various AAV serotypes in healthy humans. The sero-positivity ranged from ~72% for AAV2 and 67% for AAV1 while about 47% for AAV9, 46% for AAV6, 40% for AAV5 and 30% for AAV8 [10]. Based on the structural homology of AAV capsids, the antibodies against one serotype may cross react with other serotypes [42]. For instance, antibodies against AAV1 and AAV6 cross react efficiently indicating that they are closely related serotypes whereas AAV4, which is the most antigenically divergent serotype, is not neutralised by any other anti-sera [43,44]. AAV7 and AAV8, originally isolated from rhesus macaques, are also serologically distinct with minimal cross-reactivity to other serotypes. Of the immunoglobin subtypes, circulating levels of IgG1 are predominant followed by IgG2, IgG3 and IgG4.

In the liver-directed AAV2-hFIX clinical trial for severe Hemophilia B, one of the patients in the cohort administered with the high dose (2×10^{12} vg/kg) showed reduced FIX levels owing to his high pre treatment Nab titre (1: 17) to AAV2 [12]. A passive immunity mouse model developed to quantitatively assess Nab titres against AAV8 in male rhesus monkeys was found to be superior to *in vitro* immunoassay for detecting low Nab titres [45]. Likewise, *in vivo* assays, which are more sensitive, can be used to accurately screen patients with even low AAV-specific Nabs prior to gene transfer. In a non-human primate study to assess the impact of pre-existing immunity on AAV8-mediated gene delivery, it was demonstrated that Nab titres in excess of 1:10 substantially reduced liver transduction and caused

DOI: 10.1002/rmv

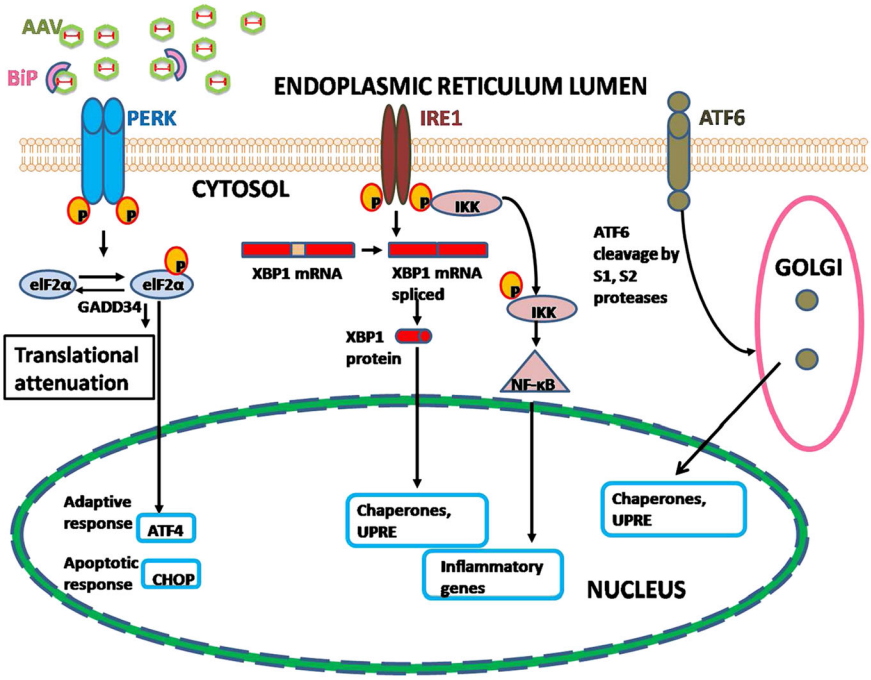

Figure 3. Unfolded protein response signalling. It is initiated by sequential and complex activation of three proteins namely (1) protein kinase R (PKR)-like ER kinase (PERK), (2) activating transcription factor 6 (ATF6) and (3) inositol-requiring enzyme 1 (IRE1), whose functions are regulated by a master regulator, immunoglobulin heavy chain binding protein (BiP). This pathway culminates in translocation of essential transcription factors into nucleus and their binding to UPREs within genes that are crucial for restoring cellular homeostasis [38]. Upon any stress such as massive influx of AAV into the ER, the stress sensors, PERK, IRE1 and ATF6, are activated. BiP, the master regulator found in the luminal domain of endoplasmic reticulum, remains attached to the sensors when the cell is in homeostasis. Under stress, BiP is released from the sensors activating them by binding to the unfolded/misfolded proteins or the viral particles. PERK dimerises and autophosphorylates and activates eIF2α by phosphorylation. Activated eIF2α represses protein translation. ATF4 downstream of PERK can escape translational repression as it has upstream open reading frames. Hence, ATF4 translocates into the nucleus activating a set of target genes necessary to bring back the cellular homeostasis or CHOP in case if the cell is in irreversible stress leading to apoptosis [32]. When IRE1 is activated, it dimerises and autophosphorylates leading to the activation of endoribonuclease activity cleaving 26 nucleotide intron from XBP1 (X-box binding protein 1) mRNA performing an unconventional splicing. Spliced XBP1 (sXBP1) protein translocates to nucleus acting as transcription factor binding to UPREs and activates many genes including chaperones that are crucial for restoring cellular homeostasis [38]. Alternatively, IRE1 interacts with TRAF2 which in turn activates IKK which phosphorylates IκB. This releases NF-κB which then translocates into the nucleus and transcribes inflammatory genes [34,35]. The third arm of UPR, ATF6 once activated by the release of BiP, translocates to Golgi complex where it is cleaved by site-1 protease (S1P) and S2P. The cleaved ATF6 fragment serves as a transcription factor enabling the transcription of chaperones and UPRE [39]

stable sequestration of capsid proteins by splenic DCs [46]. A comparative analysis of Nab titres to AAV2 and AAV8 in human subjects within the age group of 0–18 years revealed that the best age for vector infusion is between 7 and 11 months. The Nab prevalence was moderate at birth, rapidly declined during 7–11 months and then gradually increased with age thereafter. The study also suggested that after 3 years of age, AAV8 would be a better vector than AAV2 on account of its lower Nab titres [47]. Apart from humoral immunity, AAV infection at early stage of life also induces the generation of memory B cells and T cells which could be re-activated on subsequent exposure to viral vectors [7]. Screening of randomised healthy donors gave insights into immune responses to natural AAV1 infection with no correlation existing between AAV1-specific T-cell and humoral responses [48]. T-cell response was mainly centred on effector memory CD8+ cells. This finding emphasises the need to pre-screen patients for AAV-specific cellular responses, in addition to the presence of Nabs before undergoing gene therapy.

Vector-induced cellular and humoral immunity. The adaptive immune responses to AAV are directed either against the viral capsid or the encoded

transgene protein. When internalisation of the vector into host cell is followed by degradation of the viral capsid, the antigenic peptides are cross-presented to MHC I molecule [49,50] to activate CTL, which mainly orchestrate the adaptive immune reactions to AAV. A recent study [51] has elaborated on the mechanism of AAV2 capsid cross presentation and showed that it can be effectively blocked by inhibiting three major cellular processes such as endosomal acidification, proteasome system and Golgi protein transport in AAV-permissive cells. These data corroborated that the process is dependent on virion escape from the endosomes and antigen degradation by the proteasomal machinery but independent of viral uncoating in nucleus [51]. AAV is known to poorly transduce APCs, but however once internalised by these cells, they could be presented by the classical antigen-presentation pathway on either MHC I to activate CD8+ T cells or MHC II to activate CD4+ T helper cells [52]. An overview of the events associated with adaptive immune response is shown in Figure 4.

On the other hand, the transgene cassette can also influence the CD8+ T-cell reactivity [55]. The presence of certain cryptic epitopes in therapeutic FIX transgene induced CTL killing of transduced hepatocytes in mice expressing human MHC class I molecule B0702. However, no such response was observed in HLA humanised murine model when a codon-optimised transgene cassette was used in which the p18 epitope was deleted [55]. This emphasises the need to carefully analyse cDNA gene constructs for the presence of any cryptic epitopes of MHC molecules prior to gene transfer.

The differences in AAV capsid structure are known to drive variable immune response between serotypes [56]. Unlike AAV8, AAVrh32.33, a capsid variant isolated from rhesus monkey, yielded strong CTL response to both the capsid and transgene antigens [56]. Receptor affinity of the AAV capsid

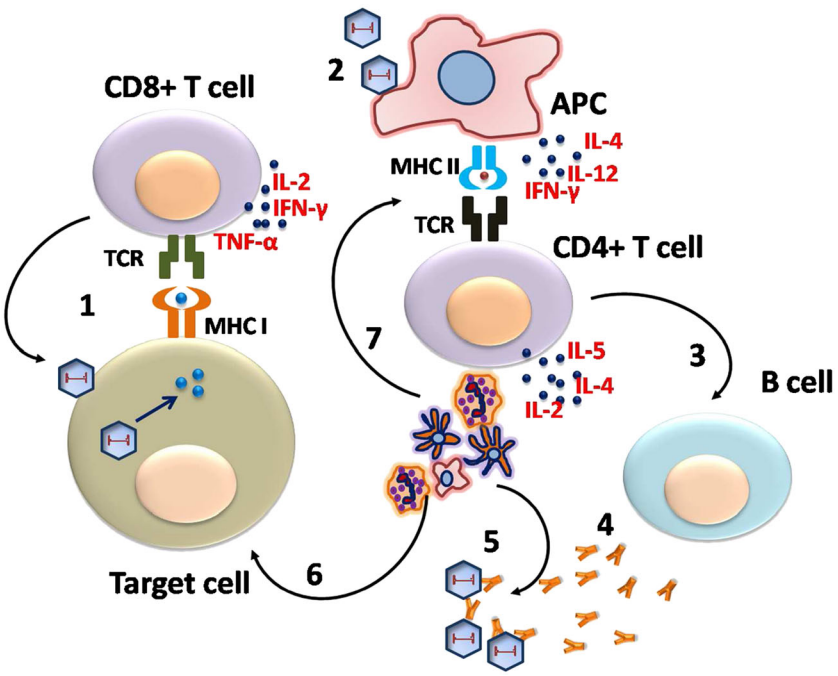

Figure 4. Adaptive immune response against AAV. (1) Transduction of target cell by recombinant AAV may be followed by the vector degradation by cellular proteasomes into antigenic peptides. These peptides then get loaded onto MHC I molecules and are presented in the cell surface to CD8+ T cells. These activated CD8+ T cells destroy the transduced host cell [52]. Activated CD8+ T cells also release pro-inflammatory cytokines such as IFNγ, TNF-α and IL-2 which can indirectly activate CD4+ T cells. (2) If AAV is captured by antigen-presenting cells (APC), they are presented through MHC II complex to T-cell receptor (TCR) of CD4+ T cells [53]. (3) These activated T cells then secrete pro-inflammatory cytokines such as IL-2, IL-4 and IL-5 to further activate B cells, in the presence of appropriate co-stimulatory signals such as CD40L also can recruit other immune cells [54]. (4) Activated B cells differentiate into plasma B cells, which secrete antibodies that specifically bind to AAV vector particles [11]. (5 and 6) Activated infiltrating immune cells like neutrophils, DCs and macrophages eliminate antibody-bound vector particles or the vector-transduced target cell. They can also serve as APCs by endocytosing AAV and amplifying the overall response (7) [53]

such as heparin binding was found to direct the T-cell response to AAV2 capsid following muscle targeted vector delivery to mice and non-human primates. Using AAV2–AAV8 hybrid vectors, a domain responsible for T-cell activation to capsid was mapped on VP3 protein. The same motif was also shown to aid in HSPG binding of the virion, speculating the role of heparin binding in uptake of AAV into DC and activation of capsid-specific T cells [57]. Other factors including dosage of vector particles administered and the route of vector infusion also determine whether immune reactions are induced or not [11,58]. The impact of route of administration on immune response induction is highlighted in the study where hepatic gene transfer protocol showed better efficacy and significantly lesser antibody formation and CD8+ T-cell infiltration compared to muscle directed delivery of FIX in mice [58]. Multiple studies also suggest that there is direct correlation between the increase in cellular vector load and immune stimulation [11,13]. In clinical trials, it was consistently observed that the patient cohort administered the highest dosage of vector particles often manifested with immune activation in due course of time, compromising the initial increase in therapeutic protein levels [11,16].

Apart from the characteristics of the vector, the native immunological status of the host also determines the nature of immune response in gene therapy. Evaluation of two different strains of mice, C57BL/6 and Balb/c, illustrated that AAV gene transfer to liver can induce tolerogenic response in the former but not in the latter. Balb/c mice fail to suppress transgene-specific CTL response but exhibit B-cell tolerance mediated by splenic Tregs [59].

Limitations of data generated from pre-clinical studies. Data from pre-clinical animal models did not predict immune-complications seen in humans. In mice, AAV capsid is shown to induce CTL response but fails to eliminate vector-transduced cells [53]. Even in immunised mice, despite the presence of pre-existing CD8+ T cells to viral capsid, the longevity of AAV-directed hepatic gene transfer of human FIX in mice was unaffected [60]. Although both humans and macaques get naturally infected with AAV, the transgene expression achieved after gene transfer is only transient in humans while sustained expression is seen in monkeys. To address such contradictions, human and macaque blood samples were tested for AAV capsid-specific T cells and subsequently analysed for T-cell subsets and differentiation markers. T cells developed in response to natural infections significantly differed between humans and non-human primates in their differentiation status and function [61]. Tolerance to AAV-encoded antigens, observed in mice during liver-directed gene delivery, was primarily due to the lack of inflammatory signals essential to render transduced hepatocytes as targets for CTL-mediated elimination [62]. Thus, studies in animal models which closely mimic the human immune system should be able to overcome this constraint. For instance, mice expressing human HLA- B0702 were used to determine that cryptic epitopes generated from ARF of FIX cDNA elicit CTL response against vector-transduced liver cells [55]. Such humanised murine and other animal models could serve in better understanding and prediction of the fate of therapeutic AAV-gene delivery with respect to its interaction with the immune system.

STRATEGIES TO EVADE IMMUNE RESPONSES AGAINST AAV VECTORS

Gene delivery to immune-privileged sites

Eye and brain, and to lesser extent liver, are considered as ideal organs for vector infusion owing to their immune-privileged status. Multiple trials have documented the ocular injections of AAV vector, to be safe and efficacious with evidence of improved visual function in the patients with LCA [63,64]. Similar safety and tolerability have been achieved with AAV in clinical trials for CNS disorders. [65]. There is also evidence to suggest that gene targeting to the liver induces immune tolerance. KC in the liver play a major role in suppressing T-cell responses during vector targeting of hepatocytes. KCs secrete anti-inflammatory molecules such as IL-10, TGF-β and prostanoids which are implicated in promoting antigenic tolerance [59]. In spite of activation of CD8+ T cells against the foreign antigen, hepatocytes escape immune destruction in the absence of additional innate immune signals controlled by TLR3 activation [66]. Long-term follow-up data from hemophilic canine and murine models have consistently proved the tolerogenic nature of the liver [58,67]. Tregs have been identified to be crucial mediators for inducing tolerance to transgene products following hepatic gene

DOI: 10.1002/rmv

transfer [59]. Antigen-specific Tregs (CD4 + CD25 + FoxP3+ T cells) suppress both cellular and humoral immune responses by way of secreting immune suppressive cytokines like IL-10 and TGF-β [68,69]. A summary of the factors which contribute to the immune-privileged status of liver upon AAV-mediated gene transfer is shown in Figure 5.

Transient immune-suppression

As a measure to attenuate or prevent unwarranted host immune responses during AAV-mediated gene therapy, various transient immune-suppression protocols have been proposed. Re-administration of AAV vector would be required to achieve sustained levels of therapeutic protein in treatment of chronic disorders like hemophilia and muscular dystrophy. However, as discussed earlier, secondary exposure to the vector entails the risk of activation of memory T cells and B cells with heightened immune reactivity and vector elimination. A short-term immune-suppression regimen induced during the initial phase of vector infusion could minimise this problem. A recent study reported that a combination of non-depleting anti-CD4 antibody and cyclosporine caused 20-fold decrease in anti-AAV8 antibody titres following intravenous administration of the AAV8 vector to immune-competent mice. This immune-suppression allowed re-administration

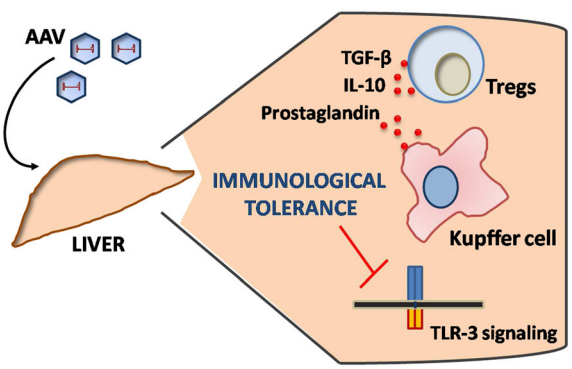

Figure 5. Factors contributing to immunological tolerance of liver during AAV gene therapy. Schematic shows that administration of AAV vectors targeting the liver results in immunological tolerance due to the various contributing factors. Tregs (CD4 + CD25 + FoxP3 + T cells) secrete immune suppressive cytokines like IL-10 and TGF-β and suppress both cellular and humoral immune responses during gene transfer to the liver [59,68,69]. KC in the liver also secrete anti-inflammatory molecules such as IL-10, TGF-β and prostanoids which promote antigenic tolerance by suppressing T-cell responses during vector targeting of hepatocytes [59]. The blockade of TLR-3 signalling in the liver further prevents the induction of CXCL9 via pro-inflammatory cytokines IFN-α and TNF-α, and thus subsequent infiltration of CD8+ T cells [66]

of the vector pseudotyped with same capsid [70]. Co-administration of mycophenolate mofetil and tacrolimus with AAV8 vector encoding human FIX appeared safe in rhesus monkeys as it did not alter the liver transduction while effectively abrogated humoral response against AAV [71]. Modulation of cell-mediated immunity using combination of CTLA-4/Ig, which blocks the T-cell priming and programmed death −1 ligands, which inhibit T effector cell function, was found to improve the immunological tolerance in muscle-directed AAV gene transfer [72]. Similarly, rituximab, a monoclonal anti-CD20 antibody targeting B cells, promoted tolerance to FVIII in gene therapy by preventing allo-antibody formation [73]. Repeated injections of AAV1 for correction of muscular dystrophy in dystrophic *mdx* mouse were evaluated with transient immunosuppression using CTLA-4/Fc and anti-mouse CD40L monoclonal antibody (MR1) which totally abrogated formation of anti-AAV1 antibodies [74]. It has been noted that proteasomal inhibition using bortezomib is also effective in reducing AAV2 capsid antigen presentation on MHC I and in moderating CTL response in mice having pre-existing immunity to AAV8 [75,76]. In the recent trial for hemophilia B using AAV8-FIX vector, two of the patients who were infused with high amounts of vector particles showed asymptomatic elevation of serum ALT levels and required a short course of glucocorticoid therapy with prednisolone to suppress this response [11]. Figure 6 shows the ligand – receptor interactions between T cell and APC and the immune-suppressive drugs that influence these interactions. These above cited examples highlight the benefits of using an immunosuppressant regimen to create a window-period for vector administration and improving the persistence of administered vectors.

Modification of AAV vectors

Several studies have shown that recombinant AAV vectors can be modified in terms of either the expression cassette or the capsid structure with the aim of mitigating cellular and humoral immunity. Restricting the expression of transgene to the target tissue would allow enhanced vector performance and prevent antigen presentation by APC and subsequent immune activation. This is done by employing tissue-specific promoters or by preventing ectopic expression using miRNAs. Sustained therapeutic protein level was reached and immune tolerance

DOI: 10.1002/rmv

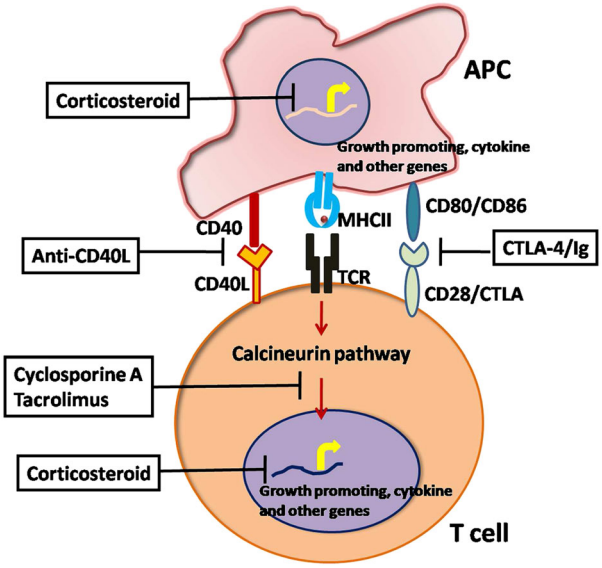

Figure 6. Immuno-suppressive agents to target APC and T-cell interactions. The interactions between various ligand and receptors of APC and T cells apart from the effect of different immuno-suppressant drugs acting at distinct steps are shown. The engagement of T-cell receptor (TCR) with peptide–MHC complex presented by APC activates calcineurin, a calcium-dependent phosphatase that activates nuclear factor of activated T cells (NFAT). This leads to increased expression of IL-2 cytokine. Cyclosporin A and tacrolimus are known to block this pathway [77]. Corticosteroids block the expression of cytokines like IL-2 and TNF-γ at the nuclear level in T cells [11] and also prevent APC differentiation and maturation [78]. T-cell activation also requires the interaction of co-stimulatory molecules on T cells and their respective ligands on APC. The interaction between CD40L and CD40 is inhibited by anti-CD40L antibody [74]. Similarly, the interaction between CD28/CTLA and CD80/CD86 is inhibited by CTLA-4/Ig [72]

induced in a fabry murine model when AAV2 vector encoding human α-galactosidase was delivered under the transcriptional control of a liver-restricted enhancer/promoter than using a ubiquitous CMV promoter [79]. Later, highly efficient liver transduction was demonstrated using scAAV vectors containing the codon-optimised human FIX gene driven by a liver-specific promoter in mice and non-human primates [80] and in human trials as well [11]. Another study reported that insertion of liver-specific miR-122 target sequence into 3′ UTR of AAV vector cassette effectively prevents the transgene expression in the liver, thus inhibiting the ectopic expression in bystander cells [81].

Capsid engineering of AAV vectors is an effective approach to evade immune response and augment their transduction potential. To shield AAV from recognition by antibodies or the T effector cells, their immunogenic epitopes may be masked. One can achieve this either by chemical or genetic alteration of specific epitopes on the capsid. Covalent attachment of synthetic polymers onto the virion surface can shield the antigen binding sites from neutralising effects of anti-AAV antibodies and circumvent the challenge of pre-existing immunity. PEGylation chemistries tested with different crosslinking groups to attach the polymer to surface lysines on the viral capsid have yielded promising results. At a critical PEG: lysine conjugation ratio, within which the vector particles are fully infective, it was noted that PEGylation rendered moderate protection against antibody neutralisation (2.3 fold) compared to unmodified AAV vectors [82,83].

Genetic modification of the capsid by mutating the Nab epitopes is an alternate way to generate immune escape phenotypes of AAV. Identification of the immunogenic epitopes was initially done by peptide scanning to map neutralising epitopes for antibodies present in mice [84] or humans [85,86]. AAV variant libraries were then generated by rational design or directed evolution (Reviewed in detail by Bartel *et al.*, 2011)[87]. Rational design utilises either insertion of peptides at specific positions that disrupt the antibody binding site of viral capsid or site-directed mutagenesis of specific residues of immunogenic peptides on AAV2 capsid [88,89]. Of the six mutants generated by insertion of a 14 amino acid peptide of the laminin fragment P1 on the VP3 protein of AAV capsid, two of them (I-534 and I-573) demonstrated ~70% reduction in affinity for human anti-sera as compared to wild-type AAV2 vectors [86]. Another study analysed single amino acid mutations on AAV2 capsid generated by site-directed mutagenesis to identify mutants with reduced antibody binding and neutralisation susceptibility [89]. Alternatively, the directed evolution approach involves generation of diverse genetic libraries of variants by either randomising the capsid by error-prone PCR mutagenesis or DNA shuffling approach to develop chimeric capsids [90–92]. The mutant variants are then subjected to high-throughput screening for infectivity in the presence of neutralising sera or T-cell activating immunogenic peptides to identify modified vectors with enhanced ability for immune evasion [91,93]. For instance, regions corresponding to immunogenic sites on AAV2 capsid were randomised to generate a viral library of approximately $6*10^6$ clones by combining rational design and evolutionary approaches, which was then selected on HEK293 cells

DOI: 10.1002/rmv

Table 1. Strategies for generating modified AAV vectors with improved transduction and immune evading phenotype

Strategy	Principle	Example	Major outcome	
			Improved transduction efficiency	*Reduced immune response*
Improved transgene cassette	Tissue-restricted promoter	Liver-specific promoter [79]	Yes	Yes
	Codon optimisation	Codon-optimised human FIX gene [80]	Yes	Yes
	Incorporation of miRNA target sequences	Liver-specific miR-122 target sequence [81]	No transgene expression in liver	
Random alteration/masking of immunogenic epitopes	Chemical modification	Conjugation of polyethylene glycol (PEG) to AAV capsid [82,83]	No	Yes
	Genetic modification	Rational design includes peptide insertion and site-directed mutagenesis [86,88,89]	No	Yes
		Directed evolution includes error-prone PCR mutagenesis and DNA shuffling [90–92]	No	Yes
Targeted capsid engineering	Site-directed mutagenesis of capsid residues	Tyrosine to phenylalanine mutation on AAV2 capsid [94]	Yes	Yes
		Serine to alanine substitution on AAV2 capsid [95]	Yes	Yes
		Lysine to arginine substitution on AAV8 capsid [96]	Yes	Yes

DOI: 10.1002/rmv

in the presence of human sera [93]. DNA shuffling method was exploited to form a library of chimeric virions of serotypes 1, 2, 4, 5, 6, 8 and 9, from which seven mutant vectors were thoroughly analysed to identify a mutant which displayed 400-fold reduction in neutralisation by IVIG [90].

Other studies with rational single amino acid modifications on AAV2 capsid have allowed generation of mutants with increased transduction or reduced reactivity to Nabs. Li *et al*. generated a mutant in which insertion of threonine at position 265 in AAV2 capsid significantly improved the vector transduction into muscles and also changed the immune profile. Furthermore, the substitution of each of the 20 amino acids at the same position produced classes of mutants with higher muscle transduction and lower Nab response, which could facilitate repeat administration of these vectors [54]. An engineered AAV2 (tyrosine to phenylalanine) mutant has been shown to minimise the CD8+ T-cell-mediated destruction of transduced hepatocytes [94]. Recently, we reported that substitution of serine to alanine residue at VP1 codon 489 on AAV2 capsid demonstrates a superior hepatic transduction in mice (14 fold) along with eight-fold reduced Nab cross-reactivity [95]. This residue was predicted to lie in one of the three phosphodegrons identified by extensive structural analysis of AAV2 capsid. Phosphodegrons are phosphorylation sites recognised as degradation sites by ubiquitin ligases and their alteration would prevent capsid degradation by the host ubiquitin/proteasomal machinery. Similarly, we demonstrated that a lysine > arginine substitution at codon 137 (K137R) lying within a phosphodegron of AAV8 capsid resulted in a mutant vector with enhanced hepatic FIX gene transfer efficiency *in vivo* and significantly reduced immunogenicity. This K137R capsid mutant displayed reduced activation of innate immune markers such as IL-6, TNF-α, IL-12, TLR9 and KC (keratinocyte chemoattractant) along with two-fold reduction in the levels of Nab than the wild-type AAV8 vector [96]. Table 1 summarises the current strategies employed to generate modified AAV vectors with an enhanced gene transfer and immune evasion potential.

CONCLUSIONS

Despite significant advancements in therapeutic gene transfer using AAV vectors, it is well recognised that several barriers related to host and vector-related immune reactions need to be overcome for long-term gene transfer. Although initially thought to be minimally immunogenic based on pre-clinical studies, data from limited human trials have highlighted the concept of vector dose-dependent immune-toxicity, consistently [11,16]. In addition, multiple factors such as the route of delivery, target organ, transgene cassette and capsid structure have been shown to evoke an immune response [56,58,97]. Striking the right balance between improved therapeutic output and lack of any immune stimulation by using optimum doses of AAV vector/or by transient immune-suppression appears to be critical. Restricting AAV transduction to only the target tissue is another major challenge. Second, the presence of and the variation in pre-existing immunity against different AAV serotypes in humans suggests that no one AAV serotype will be universally applicable for therapeutic gene transfer. Thus, it becomes important to utilise and develop other naturally occurring alternate AAV (1–10) serotypes [41,98]. In addition, any further modifications to these vectors that can improve their transduction efficiency will be desirable to counter vector dose-dependent immune response. Another confounding issue is the absence of an animal model which can accurately predict the anti-AAV immune response seen in humans [61]. However, these challenges have also provided further opportunities to dissect the biology of AAV–host cellular interactions. The renaissance of AAV-mediated gene therapy over the last five years has offered renewed hope that these challenges will be conquered soon.

CONFLICT OF INTEREST

There are no conflicts of interest for any of the authors.

ACKNOWLEDGEMENTS

SH is supported by a senior research fellowship from Council for Scientific and Industrial research (CSIR), Government of India. BB is supported by a junior research fellowship from University Grants Commission (UGC), Government of India. GRJ is supported by research grants from Department of Science of Technology, Government of India (Swarnajayanti Fellowship 2011), Department of Biotechnology (DBT), Government of India (Innovative Young Biotechnologist award 2010: BT/03/IYBA/2010; Grant: BT/PR14748/MED/12/491/2010; Grant: BT/01/COE/08/03) and an early career investigator award-2010 from Bayer Hemophilia Awards program, Bayer Inc, USA.

REFERENCES

1. Asokan A, Schaffer DV, Samulski RJ. The AAV vector toolkit: poised at the clinical crossroads. *Molecular Therapy* 2012; **20**: 699–708.
2. Lundberg C, Bjorklund T, Carlsson T, *et al*. Applications of lentiviral vectors for biology and gene therapy of neurological disorders. *Current Gene Therapy* 2008; **8**: 461–473.
3. Yi Y, Noh MJ, Lee KH. Current advances in retroviral gene therapy. *Current Gene Therapy* 2011; **11**: 218–228.
4. Zaiss AK, Liu Q, Bowen GP, Wong NC, Bartlett JS, Muruve DA. Differential activation of innate immune responses by adenovirus and adeno-associated virus vectors. *Journal of Virology* 2002; **76**: 4580–4590.
5. Daya S, Berns KI. Gene therapy using adeno-associated virus vectors. *Clinical Microbiology Reviews* 2008; **21**: 583–593.
6. Wright JF. Transient transfection methods for clinical adeno-associated viral vector production. *Human Gene Therapy* 2009; **20**: 698–706.
7. Mingozzi F, High KA. Therapeutic in vivo gene transfer for genetic disease using AAV: progress and challenges. *Nature Reviews Genetics* 2011; **12**: 341–355.
8. Zincarelli C, Soltys S, Rengo G, Rabinowitz JE. Analysis of AAV serotypes 1–9 mediated gene expression and tropism in mice after systemic injection. *Molecular Therapy* 2008; **16**: 1073–1080.
9. Mendell JR, Campbell K, Rodino-Klapac L, *et al*. Dystrophin immunity in Duchenne's muscular dystrophy. *New England Journal of Medicine* 2010; **363**: 1429–1437.
10. Boutin S, Monteilhet V, Veron P, *et al*. Prevalence of serum IgG and neutralizing factors against adeno-associated virus (AAV) types 1, 2, 5, 6, 8, and 9 in the healthy population: implications for gene therapy using AAV vectors. *Human Gene Therapy* 2010; **21**: 704–712.
11. Nathwani AC, Tuddenham EG, Rangarajan S, *et al*. Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *New England Journal of Medicine* 2011; **365**: 2357–2365.
12. Manno CS, Pierce GF, Arruda VR, *et al*. Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nature Medicine* 2006; **12**: 342–347.
13. Mingozzi F, Maus MV, Hui DJ, *et al*. CD8(+) T-cell responses to adeno-associated virus capsid in humans. *Nature Medicine* 2007; **13**: 419–422.
14. McPhee SW, Janson CG, Li C, *et al*. Immune responses to AAV in a phase I study for Canavan disease. *The Journal of Gene Medicine* 2006; **8**: 577–588.
15. Brantly ML, Chulay JD, Wang L, *et al*. Sustained transgene expression despite T lymphocyte responses in a clinical trial of rAAV1-AAT gene therapy. *Proceedings of the National Academy of Sciences of the United States of America* 2009; **106**: 16363–16368.
16. Mingozzi F, Meulenberg JJ, Hui DJ, *et al*. AAV-1-mediated gene transfer to skeletal muscle in humans results in dose-dependent activation of capsid-specific T cells. *Blood* 2009; **114**: 2077–2086.
17. Jooss K, Ertl HC, Wilson JM. Cytotoxic T-lymphocyte target proteins and their major histocompatibility complex class I restriction in response to adenovirus vectors delivered to mouse liver. *Journal of Virology* 1998; **72**: 2945–2954.
18. Zaiss AK, Muruve DA. Immunity to adeno-associated virus vectors in animals and humans: a continued challenge. *Gene Therapy* 2008; **15**: 808–816.
19. Zhang Y, Chirmule N, Gao G, Wilson J. CD40 ligand-dependent activation of cytotoxic T lymphocytes by adeno-associated virus vectors in vivo: role of immature dendritic cells. *Journal of Virology* 2000; **74**: 8003–8010.
20. Jooss K, Yang Y, Fisher KJ, Wilson JM. Transduction of dendritic cells by DNA viral vectors directs the immune response to transgene products in muscle fibers. *Journal of Virology* 1998; **72**: 4212–4223.
21. Zaiss AK, Cotter MJ, White LR, *et al*. Complement is an essential component of the immune response to adeno-associated virus vectors. *Journal of Virology* 2008; **82**: 2727–2740.
22. Bartlett JS, Wilcher R, Samulski RJ. Infectious entry pathway of adeno-associated virus and adeno-associated virus vectors. *Journal of Virology* 2000; **74**: 2777–2785.
23. Martino AT, Suzuki M, Markusic DM, *et al*. The genome of self-complementary adeno-associated viral vectors increases Toll-like receptor 9-dependent innate immune responses in the liver. *Blood* 2011; **117**: 6459–6468.
24. Jayandharan GR, Aslanidi G, Martino AT, *et al*. Activation of the NF-kappaB pathway by adeno-associated virus (AAV) vectors and its implications in immune response and gene therapy. *Proceedings of the National Academy of Sciences of the United States of America* 2011; **108**: 3743–3748.
25. Balakrishnan B, Sen D, Hareendran S, *et al*. Activation of the cellular unfolded protein response by recombinant adeno-associated virus vectors. *PLoS One* 2013; **8**: e53845.
26. Mogensen TH. Pathogen recognition and inflammatory signaling in innate immune defenses. *Clinical Microbiology Reviews* 2009; **22**: 240–273.
27. Zhu J, Huang X, Yang Y. The TLR9-MyD88 pathway is critical for adaptive immune responses to adeno-associated virus gene therapy vectors in mice. *Journal of Clinical Investigation* 2009; **119**: 2388–2398.
28. Rogers GL, Martino AT, Aslanidi GV, Jayandharan GR, Srivastava A, Herzog RW. Innate Immune Responses to AAV Vectors. *Frontiers in Microbiology* 2011; **2**: 194.
29. Ding W, Zhang L, Yan Z, Engelhardt JF. Intracellular trafficking of adeno-associated viral vectors. *Gene Therapy* 2005; **12**: 873–880.
30. Nonnenmacher M, Weber T. Intracellular transport of recombinant adeno-associated virus vectors. *Gene Therapy* 2012; **19**: 649–658.
31. Schroder M, Kaufman RJ. The mammalian unfolded protein response. *Annual Review of Biochemistry* 2005; **74**: 739–789.
32. Harding HP, Zhang Y, Ron D. Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. *Nature* 1999; **397**: 271–274.
33. Costa CZ, da Rosa SE, de Camargo MM. The unfolded protein response: how protein folding became a restrictive aspect for innate immunity and B lymphocytes. *Scandinavian Journal of Immunology* 2011; **73**: 436–448.
34. Kaneko M, Niinuma Y, Nomura Y. Activation signal of nuclear factor-kappa B in response to

endoplasmic reticulum stress is transduced via IRE1 and tumor necrosis factor receptor-associated factor 2. *Biological and Pharmaceutical Bulletin* 2003; **26**: 931–935.

35. Hu P, Han Z, Couvillon AD, Kaufman RJ, Exton JH. Autocrine tumor necrosis factor alpha links endoplasmic reticulum stress to the membrane death receptor pathway through IRE1alpha-mediated NF-kappaB activation and down-regulation of TRAF2 expression. *Molecular and Cellular Biology* 2006; **26**: 3071–3084.
36. Summerford C, Samulski RJ. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *Journal of Virology* 1998; **72**: 1438–1445.
37. Gu F, Crump CM, Thomas G. Trans-Golgi network sorting. *Cellular and Molecular Life Sciences* 2001; **58**: 1067–1084.
38. Calfon M, Zeng H, Urano F, *et al*. IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature* 2002; **415**: 92–96.
39. Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell* 2001; **107**: 881–891.
40. Bossis I, Chiorini JA. Cloning of an avian adeno-associated virus (AAAV) and generation of recombinant AAAV particles. *Journal of Virology* 2003; **77**: 6799–6810.
41. Gao GP, Alvira MR, Wang L, Calcedo R, Johnston J, Wilson JM. Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proceedings of the National Academy of Sciences of the United States of America* 2002; **99**: 11854–11859.
42. Calcedo R, Vandenberghe LH, Gao G, Lin J, Wilson JM. Worldwide epidemiology of neutralizing antibodies to adeno-associated viruses. *Journal of Infectious Diseases* 2009; **199**: 381–390.
43. Harbison CE, Weichert WS, Gurda BL, Chiorini JA, Agbandje-McKenna M, Parrish CR. Examining the cross-reactivity and neutralization mechanisms of a panel of mAbs against adeno-associated virus serotypes 1 and 5. *Journal of General Virology* 2012; **93**: 347–355.
44. Grimm D, Zhou S, Nakai H, *et al*. Preclinical in vivo evaluation of pseudotyped adeno-associated virus vectors for liver gene therapy. *Blood* 2003; **102**: 2412–2419.
45. Sun L, Tu L, Gao G, Sun X, Duan J, Lu Y. Assessment of a passive immunity mouse model to quantitatively analyze the impact of neutralizing antibodies on adeno-associated virus-mediated gene transfer. *Journal of Immunological Methods* 2013; **387**: 114–120.
46. Wang L, Calcedo R, Bell P, *et al*. Impact of pre-existing immunity on gene transfer to nonhuman primate liver with adeno-associated virus 8 vectors. *Human Gene Therapy* 2011; **22**: 1389–1401.
47. Calcedo R, Morizono H, Wang L, *et al*. Adeno-associated virus antibody profiles in newborns, children, and adolescents. *Clinical and Vaccine Immunology* 2011; **18**: 1586–1588.
48. Veron P, Leborgne C, Monteilhet V, *et al*. Humoral and cellular capsid-specific immune responses to adeno-associated virus type 1 in randomized healthy donors. *Journal of Immunology* 2012; **188**: 6418–6424.
49. Burgdorf S, Scholz C, Kautz A, Tampe R, Kurts C. Spatial and mechanistic separation of cross-presentation and endogenous antigen presentation. *Nature Immunology* 2008; **9**: 558–566.
50. Ramachandra L, Simmons D, Harding CV. MHC molecules and microbial antigen processing in phagosomes. *Current Opinion in Immunology* 2009; **21**: 98–104.
51. Li C, He Y, Nicolson S, *et al*. Adeno-associated virus capsid antigen presentation is dependent on endosomal escape. *Journal of Clinical Investigation* 2013; **123**: 1390–1401.
52. Pien GC, Basner-Tschakarjan E, Hui DJ, *et al*. Capsid antigen presentation flags human hepatocytes for destruction after transduction by adeno-associated viral vectors. *Journal of Clinical Investigation* 2009; **119**: 1688–1695.
53. Li C, Hirsch M, Asokan A, *et al*. Adeno-associated virus type 2 (AAV2) capsid-specific cytotoxic T lymphocytes eliminate only vector-transduced cells coexpressing the AAV2 capsid in vivo. *Journal of Virology* 2007; **81**: 7540–7547.
54. Li C, Diprimio N, Bowles DE, *et al*. Single amino acid modification of adeno-associated virus capsid changes transduction and humoral immune profiles. *Journal of Virology* 2012; **86**: 7752–7759.
55. Li C, Goudy K, Hirsch M, *et al*. Cellular immune response to cryptic epitopes during therapeutic gene transfer. *Proceedings of the National Academy of Sciences of the United States of America* 2009; **106**: 10770–10774.
56. Mays LE, Vandenberghe LH, Xiao R, *et al*. Adeno-associated virus capsid structure drives CD4-dependent CD8+ T cell response to vector encoded proteins. *Journal of Immunology* 2009; **182**: 6051–6060.
57. Vandenberghe LH, Wang L, Somanathan S, *et al*. Heparin binding directs activation of T cells against adeno-associated virus serotype 2 capsid. *Nature Medicine* 2006; **12**: 967–971.
58. Cao O, Hoffman BE, Moghimi B, *et al*. Impact of the underlying mutation and the route of vector administration on immune responses to factor IX in gene therapy for hemophilia B. *Molecular Therapy* 2009; **17**: 1733–1742.
59. Breous E, Somanathan S, Vandenberghe LH, Wilson JM. Hepatic regulatory T cells and Kupffer cells are crucial mediators of systemic T cell tolerance to antigens targeting murine liver. *Hepatology* 2009; **50**: 612–621.
60. Li H, Murphy SL, Giles-Davis W, *et al*. Pre-existing AAV capsid-specific CD8+ T cells are unable to eliminate AAV-transduced hepatocytes. *Molecular Therapy* 2007; **15**: 792–800.
61. Li H, Lasaro MO, Jia B, *et al*. Capsid-specific T-cell responses to natural infections with adeno-associated viruses in humans differ from those of nonhuman primates. *Molecular Therapy* 2011; **19**: 2021–2030.
62. Somanathan S, Breous E, Bell P, Wilson JM. AAV vectors avoid inflammatory signals necessary to render transduced hepatocyte targets for destructive T cells. *Molecular Therapy* 2010; **18**: 977–982.
63. Maguire AM, Simonelli F, Pierce EA, *et al*. Safety and efficacy of gene transfer for Leber's congenital amaurosis. *New England Journal of Medicine* 2008; **358**: 2240–2248.
64. Hauswirth WW, Aleman TS, Kaushal S, *et al*. Treatment of leber congenital amaurosis due to RPE65 mutations by ocular subretinal injection of adeno-associated virus gene vector: short-term results of a

phase I trial. *Human Gene Therapy* 2008; **19**: 979–990.

65. LeWitt PA, Rezai AR, Leehey MA, *et al*. AAV2-GAD gene therapy for advanced Parkinson's disease: a double-blind, sham-surgery controlled, randomised trial. *Lancet Neurology* 2011; **10**: 309–319.
66. Lang KS, Georgiev P, Recher M, *et al*. Immunoprivileged status of the liver is controlled by Toll-like receptor 3 signaling. *Journal of Clinical Investigation* 2006; **116**: 2456–2463.
67. Niemeyer GP, Herzog RW, Mount J, *et al*. Long-term correction of inhibitor-prone hemophilia B dogs treated with liver-directed AAV2-mediated factor IX gene therapy. *Blood* 2009; **113**: 797–806.
68. Kelly ME, Zhuo J, Bharadwaj AS, Chao H. Induction of immune tolerance to FIX following muscular AAV gene transfer is AAV-dose/FIX-level dependent. *Molecular Therapy* 2009; **17**: 857–863.
69. Hoffman BE, Martino AT, Sack BK, *et al*. Nonredundant roles of IL-10 and TGF-beta in suppression of immune responses to hepatic AAV-factor IX gene transfer. *Molecular Therapy* 2011; **19**: 1263–1272.
70. McIntosh JH, Cochrane M, Cobbold S, *et al*. Successful attenuation of humoral immunity to viral capsid and transgenic protein following AAV-mediated gene transfer with a non-depleting CD4 antibody and cyclosporine. *Gene Therapy* 2012; **19**: 78–85.
71. Jiang H, Lillicrap D, Patarroyo-White S, *et al*. Multiyear therapeutic benefit of AAV serotypes 2, 6, and 8 delivering factor VIII to hemophilia A mice and dogs. *Blood* 2006; **108**: 107–115.
72. Adriouch S, Franck E, Drouot L, *et al*. Improved Immunological Tolerance Following Combination Therapy with CTLA-4/Ig and AAV-Mediated PD-L1/2 Muscle Gene Transfer. *Frontiers in Microbiology* 2011; **2**: 199.
73. Sack BK, Merchant S, Markusic DM, *et al*. Transient B cell depletion or improved transgene expression by codon optimization promote tolerance to factor VIII in gene therapy. *PLoS One* 2012; **7**: e37671.
74. Lorain S, Gross DA, Goyenvalle A, Danos O, Davoust J, Garcia L. Transient immunomodulation allows repeated injections of AAV1 and correction of muscular dystrophy in multiple muscles. *Molecular Therapy* 2008; **16**: 541–547.
75. Karman J, Gumlaw NK, Zhang J, Jiang JL, Cheng SH, Zhu Y. Proteasome inhibition is partially effective in attenuating pre-existing immunity against recombinant adeno-associated viral vectors. *PLoS One* 2012; **7**: e34684.
76. Finn JD, Hui D, Downey HD, *et al*. Proteasome inhibitors decrease AAV2 capsid derived peptide epitope presentation on MHC class I following transduction. *Molecular Therapy* 2010; **18**: 135–142.
77. O'Neill LA. Targeting signal transduction as a strategy to treat inflammatory diseases. *Nature Reviews Drug Discovery* 2006; **5**: 549–563.
78. Hackstein H, Thomson AW. Dendritic cells: emerging pharmacological targets of immunosuppressive drugs. *Nature Reviews Immunology* 2004; **4**: 24–34.
79. Ziegler RJ, Lonning SM, Armentano D, *et al*. AAV2 vector harboring a liver-restricted promoter facilitates sustained expression of therapeutic levels of alpha-galactosidase A and the induction of immune tolerance in Fabry mice. *Molecular Therapy* 2004; **9**: 231–240.
80. Nathwani AC, Gray JT, Ng CY, *et al*. Self-complementary adeno-associated virus vectors containing a novel liver-specific human factor IX expression cassette enable highly efficient transduction of murine and nonhuman primate liver. *Blood* 2006; **107**: 2653–2661.
81. Qiao C, Yuan Z, Li J, *et al*. Liver-specific microRNA-122 target sequences incorporated in AAV vectors efficiently inhibits transgene expression in the liver. *Gene Therapy* 2011; **18**: 403–410.
82. Lee GK, Maheshri N, Kaspar B, Schaffer DV. PEG conjugation moderately protects adeno-associated viral vectors against antibody neutralization. *Biotechnology and Bioengineering* 2005; **92**: 24–34.
83. Le HT, Yu QC, Wilson JM, Croyle MA. Utility of PEGylated recombinant adeno-associated viruses for gene transfer. *Journal of Controlled Release* 2005; **108**: 161–177.
84. Zhong L, Li B, Mah CS, *et al*. Next generation of adeno-associated virus 2 vectors: point mutations in tyrosines lead to high-efficiency transduction at lower doses. *Proceedings of the National Academy of Sciences of the United States of America* 2008; **105**: 7827–7832.
85. Moskalenko M, Chen L, van Roey M, *et al*. Epitope mapping of human anti-adeno-associated virus type 2 neutralizing antibodies: implications for gene therapy and virus structure. *Journal of Virology* 2000; **74**: 1761–1766.
86. Huttner NA, Girod A, Perabo L, *et al*. Genetic modifications of the adeno-associated virus type 2 capsid reduce the affinity and the neutralizing effects of human serum antibodies. *Gene Therapy* 2003; **10**: 2139–2147.
87. Bartel M, Schaffer D, Buning H. Enhancing the Clinical Potential of AAV Vectors by Capsid Engineering to Evade Pre-Existing Immunity. *Frontiers in Microbiology* 2011; **2**: 204.
88. Wobus CE, Hugle-Dorr B, Girod A, Petersen G, Hallek M, Kleinschmidt JA. Monoclonal antibodies against the adeno-associated virus type 2 (AAV-2) capsid: epitope mapping and identification of capsid domains involved in AAV-2-cell interaction and neutralization of AAV-2 infection. *Journal of Virology* 2000; **74**: 9281–9293.
89. Lochrie MA, Tatsuno GP, Christie B, *et al*. Mutations on the external surfaces of adeno-associated virus type 2 capsids that affect transduction and neutralization. *Journal of Virology* 2006; **80**: 821–834.
90. Koerber JT, Klimczak R, Jang JH, Dalkara D, Flannery JG, Schaffer DV. Molecular evolution of adeno-associated virus for enhanced glial gene delivery. *Molecular Therapy* 2009; **17**: 2088–2095.
91. Maheshri N, Koerber JT, Kaspar BK, Schaffer DV. Directed evolution of adeno-associated virus yields enhanced gene delivery vectors. *Nature Biotechnology* 2006; **24**: 198–204.
92. Perabo L, Buning H, Kofler DM, *et al*. In vitro selection of viral vectors with modified tropism: the adeno-associated virus display. *Molecular Therapy* 2003; **8**: 151–157.
93. Maersch S, Huber A, Buning H, Hallek M, Perabo L. Optimization of stealth adeno-

associated virus vectors by randomization of immunogenic epitopes. *Virology* 2009; **397**: 167–175.

94. Martino AT, Basner-Tschakarjan E, Markusic DM, *et al*. Engineered AAV vector minimizes in vivo targeting of transduced hepatocytes by capsid-specific CD8+ T cells. *Blood* 2013; **121**: 2224–2233.
95. Gabriel N, Hareendran S, Sen D, *et al*. Bioengineering of AAV2 Capsid at Specific Serine, Threonine, or Lysine Residues Improves Its Transduction Efficiency in Vitro and in Vivo. *Human Gene Therapy Methods* 2013; **24**: 80–93.
96. Sen D, Gadkari RA, Sudha G, *et al*. Targeted modifications in adeno-associated virus (AAV) serotype −8 capsid improves its hepatic gene transfer efficiency in vivo. *Human Gene Therapy Methods* 2013; **24**: 104–106.
97. Breous E, Somanathan S, Wilson JM. BALB/c mice show impaired hepatic tolerogenic response following AAV gene transfer to the liver. *Molecular Therapy* 2010; **18**: 766–774.
98. Gao G, Vandenberghe LH, Alvira MR, *et al*. Clades of Adeno-associated viruses are widely disseminated in human tissues. *Journal of Virology* 2004; **78**: 6381–6388.