Cellular Immunology 342 (2019) 103658

Contents lists available at ScienceDirect

Cellular Immunology

journal homepage: www.elsevier.com/locate/ycimm

Review article

Complexity of immune responses to AAV transgene products – Example of factor IX

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ARTICLE INFO

Keywords:
Adeno-associated virus
Hemophilia
Gene therapy
Antibody
T cell
Tolerance

ABSTRACT

After two decades of research, *in vivo* gene transfer with adeno-associated viral (AAV) vectors has now resulted in successful treatments and even cures for several human diseases. However, the potential for immune responses against the therapeutic gene products remains one of the concerns as this approach is broadened to more patients, diverse diseases, and target organs. Immune responses following gene transfer of coagulation factor IX (FIX) for the treatment of the bleeding disorder hemophilia B has been extensively investigated in multiple animal models. Findings from these studies have not only influenced clinical trial design but have broader implications for other diseases. The impact of vector design and dose, as well as target organ/route of administration on humoral and cellular immune responses are reviewed. Furthermore, the potential for tolerance induction by hepatic gene transfer or combination with immune modulation is discussed.

1. Introduction

The potential of adeno-associated viral (AAV) vectors to achieve long-term transgene gene expression without immune rejection after *in vivo* gene transfer came to light in a series of publications in 1996, demonstrating a lack of cytotoxic T lymphocyte (CTL) responses that had plagued adenoviral vectors [1–3]. Subsequent studies have shown that immune responses to the transgene product remain a complex problem to solve and are impacted by many factors [4]. Thus far, such responses have been rare in clinical trials (although, cases of $CD8^+$ T cell responses against dystrophin and α_1-antitrypsin have been documented upon intramuscular administration of AAV vectors) [5,6]. However, patients have also been carefully selected in many trials. For example, only patients with extensive prior exposure to factor VIII (FVIII) or factor IX (FIX) without history of antibody formation in protein replacement therapy are included in the gene therapy trials for hemophilia A and B, respectively, and only hemophilia B patients with F9 missense mutations received muscle gene transfer [7–9]. In some cases, such as ocular gene transfer, small amounts of vector may be given to an immune privileged site [10–12]. Furthermore, pre-clinical studies in animal models have help guide clinical trial design to minimize the risk for unwanted immune responses [13]. Nonetheless, avoiding for example antibody formation against secreted transgene products remains a critical and active field of investigation. Many of these questions gain further importance as the field moves toward systemic delivery of vector in order to achieve therapy in multiple organs. This review primarily focuses on *in vivo* administration of AAV vectors expressing coagulation factor IX (FIX) for the treatment of the bleeding disorder hemophilia B as an example for the complexity and questions that arise when attempting to prevent cellular and humoral immune responses to systemically delivered transgene products. For example, transgene products may be secreted into the systemic circulation to correct a missing function in the blood, as in the case of FIX, or systemically delivered to multiple organs, as in the case of storage disorders or muscular dystrophies. Important questions include: What determines the risk of immune responses? How can the gene transfer protocol be designed to reduce such risks? Which are the mechanisms that regulate immune responses? Are immune suppression protocols helpful? Can immune tolerance be achieved?

2. Antibody formation against secreted gene products such as factor IX

In contrast to intravenous delivery of therapeutic proteins, which requires frequent injections due to limited half-life in circulation, gene therapy can correct inherited protein deficiencies long-term. However, a major concern in gene therapy for such diseases is the potential for antibody formation against the transgene product, which would then also render the patient's conventional protein therapy ineffective. This problem has been studied extensively in the context of gene therapy for the X-linked bleeding disorder hemophilia. The inability of a hemophilic patient's blood to clot is caused by mutations in FIX (a serine protease), resulting in hemophilia B, or its crucial co-factor (FVIII), resulting in hemophilia A. Currently, patients with hemophilia A or B

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http://dx.doi.org/10.1016/j.cellimm.2017.05.006
Received 19 April 2017; Received in revised form 26 May 2017; Accepted 27 May 2017
Available online 29 May 2017

Table 1
Overview of liver-directed AAV gene therapy trials (active, recruiting patients, or pending, as of May 2017).

Identifier	Status	Sponsor	Vector	Dose (vg/kg)
Hemophilia A				
NCT02576795	Recruiting	BioMarin	AAV5-F8 (BMN 270)	6×10^{12}–6×10^{13}
NCT03003533	Recruiting	Spark Therapeutics	Engineered AAV-F8 (SPK-8011)	Not disclosed
NCT03001830	Pending	University College London	AAV8-F8	6×10^{11}–6×10^{12}
NCT03061201	Pending	Sangamo Biosciences	AAV6-F8	Not disclosed
Hemophilia B				
NCT01687608	Active	Baxalta	scAAV8-coF9 (BAX 335/AskBio009)	Not disclosed
NCT02396342	Active	UniQure Biopharma B.V.	AAV5-F9	5×10^{12}–2×10^{13}
NCT02618915	Active	Dimension Therapeutics	AAVrh10-F9	1.6×10^{12}–1×10^{13}
NCT00979238	Recruiting	St. Jude Children's Research Hospital	AAV2/8–coF9	2×10^{11}–2×10^{12}
NCT02484092	Recruiting	Spark Therapeutics	Engineered AAV-coF9 (SPK9001)	Not disclosed
NCT02695160	Recruiting	Sangamo Biosciences	AAV2/6-(ZFN1, ZFN2, F9)	5×10^{12}–5×10^{13}
NCT02971969	Recruiting	Dimension Therapeutics	AAVrh10-F9 (LTFU)	1.6×10^{12}–1×10^{13}

are predominantly treated by intravenous infusion of FVIII or FIX concentrate. Gene therapy has always been an attractive alternative and aims to transfer a functional F8 or F9 gene to target cells that are capable of producing biologically active proteins and of secreting these at sufficient levels into the blood [14]. Raising coagulation activity to > 1% would change the disease phenotype from severe to moderate, while raising levels to > 5% would result in a mild phenotype. The goal is to achieve stable gene transfer in order to restore hemostasis long-term, thereby preventing bleeds, which occur frequently in severe patients, cause tissue damage and pain, and can be life threatening if left untreated.

FVIII and FIX are normally synthesized in the liver by endothelial cells and hepatocytes, respectively [14]. However, other cell types are capable of producing biologically active FVIII and FIX molecules, providing a wide range of potential target cells and tissues for gene therapy. Moreover, precise regulation of gene expression is not required. Recent gene therapy trials utilized AAV vectors for gene transfer to hepatocytes to correct hemophilia A and B [7,8]. After initial setbacks, this approach has now been successful in a number of patients, resulting even in complete correction of disease [7,8,15]. Table 1 summarizes current trials on hepatic gene transfer. At the 2017 annual meeting of the American Society of Gene and Cell Therapy (Washington, DC), one trial reported sustained FIX activity levels of approximately 30% of normal (on average) in 10 patients using a dose of 5×10^{11} vector genomes (vg) per kg of an AAV vector with an engineered capsid expressing the hyperactive FIX-Padua variant (NCT02484092). Another trial (NCT02576795), expressing a codon-optimized FVIII sequence, had reported sustained levels of > 100% of normal in multiple patients after high-dose gene transfer (6×10^{13} vg/kg) of an AAV5 vector (presented at the 2016 World Federation of Hemophilia meeting, Orlando, Florida).

A major complication of the traditional, protein-based therapy that gene therapy seeks to replace is the formation of neutralizing anti-drug antibodies, termed "inhibitors" [16,17]. Inhibitors are antibodies against FVIII or FIX that inhibit coagulation activity. Their presence in plasma samples is detected, and their titers are measured *in vitro* using the Bethesda assay. Inhibitor formation occurs in 25–30% of severe hemophilia A patients (i.e. patients with < 1% coagulation activity) and in a smaller portion of hemophilia B patients (3–5%). FIX inhibitors may be associated with additional immunotoxicities such as anaphylactic reactions. Although there are bypass agents to treat inhibitor patients, these treatments are more challenging and also very expensive. Inhibitor patients are considered at higher risk for morbidity and mortality. It is therefore critical to develop gene therapy protocols with minimal risk of inhibitor formation.

3. Multiple factors impact the risk of immune responses to the transgene product

3.1. Complexity of the risk of immune responses

Inhibitor formation is a T help-dependent B cell response. Approximately 20 years ago, when AAV-based gene therapies for hemophilia were first being developed, experiments primarily focused on FIX expression. The FIX cDNA is small compared to FVIII, so that it can be incorporated in the AAV genome under the control of a strong promoter. FIX is also better secreted and can be more easily produced in many expression systems. However, the risk of unwanted immune responses in a gene therapy setting was unknown. AAV vectors efficiently transfer genes to different types of non-dividing cells *in vivo*, including skeletal muscle [18]. The non-invasiveness of intramuscular injections and the fact that major organs were avoided made muscle-directed gene transfer attractive as a first, fairly safe target for AAV-FIX gene transfer to patients with severe hemophilia B [19]. Over the course of a decade, immune responses to FIX were studied in AAV gene therapy using murine and canine models of hemophilia B [20]. In addition to antibody formation, cytotoxic T lymphocyte (CTL) responses are a concern in gene therapy, since such activated $CD8^+$ T cells can eliminate transduced cells that display transgene product-derived peptides on the cell surface via MHC I (Fig. 1).

The sum of these studies indicates that the risk of immune responses, both antibody-mediated and cellular, against a FIX transgene product varies substantially depending on a number of factors. These include the choice of vector, specific vector design, target tissue/route of administration/specific design of the gene transfer protocol, vector dose, the underlying F9 mutation, and other host factors. For example, adenovirus activates much more efficiently $CD8^+$ T cells than AAV, resulting in CTL responses, which is further enhanced by stronger Th1 responses [21]. Higher transduction of professional antigen presenting cells (APCs) by adenovirus and more potent innate immune signals from adenoviral infection contribute to this difference [22]. The risk of CTL responses can be reduced by elimination of viral gene products (which is the case for all AAV vectors) and by use of a tightly regulated cell-specific promoter for the transgene to avoid expression in APCs (Fig. 1) [23]. In addition, microRNA targets can be incorporated into the transgene mRNA to cause its degradation in APCs [24]. Nonetheless, there is a residual risk of $CD8^+$ T cell activation due to cross-presentation of the antigen, which involves the uptake of the transgene product by APCs followed by re-routing into the MHC I presentation pathway [25–27]. Furthermore, avoiding expression in APCs is less likely to prevent antibody formation, which is the result of MHC II presentation of the transgene product taken up by APCs (Fig. 1). The extent to which $CD8^+$ T cell vs B cell responses may occur is also influenced by intrinsic properties of the antigen and thus may differ for

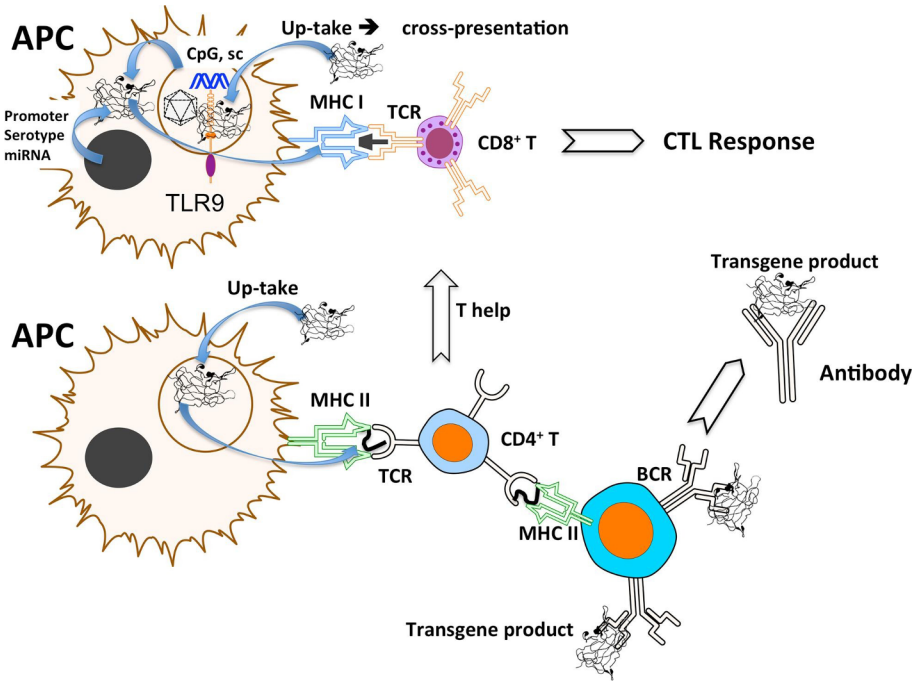

Fig. 1. Mechanisms of antibody and CD8$^+$ T cell responses to the transgene product in AAV gene transfer. CD8$^+$ T cell activation is dependent on innate signals derived from TLR9 sensing of the AAV genome. Several methods can minimize expression of the transgene product in professional antigen presenting cells (APCs), such as use of promoters that do not express well in APCs, incorporation of miRNA targets in the expression cassette, and use of serotypes that fail to transduce APCs. None of these approaches, however, prevent cross-presentation. TCR: T cell receptor; BAR: B cell receptor; MHC: major histocompatibility complex; TLR9: toll-like receptor 9.

various transgene products even if the gene transfer protocol is identical. Host genetics also play a role. For example, CD8$^+$ T cell responses against FIX are typically absent after muscle gene transfer to C57BL/6 mice but may be seen in other strains [21,28–31].

3.2. Elevated risk of immune response in muscle gene transfer

Multiple animal studies found that gene transfer to skeletal muscle poses an elevated risk of inducing immune responses to the transgene product [32]. Systemic delivery of a transgene product secreted from muscle fibers is limited by a local immune response, resulting in T and B cell activation in draining lymph nodes, and eventually developing into a systemic immune response [26,31,32]. Transgene product-specific CD4$^+$ and CD8$^+$ T cells activated in the draining lymph node migrate to the site of expression in the muscle and participate in the ensuing inflammatory response. However, the inflammatory T cell response often fails to eliminate the transduced fibers, and the inflammation may resolve over the course of several months when using single-stranded AAV vectors [31]. In this case, activated CD8$^+$ T cells express negative checkpoint markers such as PD-1 and undergo apoptotic cell death in the muscle [33,34]. In contrast, self-complementary vectors induce more functional CD8$^+$ T cell responses [30,35]. This may in part relate to differences in kinetics and levels of transgene expression. Furthermore, innate immune sensing of the AAV genome by the endosomal DNA receptor TLR9 is a critical signal for the activation of CD8$^+$ T cell responses to the transgene product in skeletal muscle [36,37]. Using self-complementary vectors increases this signal, while elimination of immune stimulatory CpG motifs (which, in contrast to mammalian DNA, lack methylation in bacterial and viral genomes) reduces CD8$^+$ T cell activation (while, however, having little effect on antibody formation) [36,38]. TLR9-MyD88 signaling has some modulatory effect on Th1 vs Th2 activation and therefore the ratio of different immunoglobulin subclasses that are produced against the AAV capsid and the transgene product, and there are examples of heightened antibody responses when using scAAV vectors [30,37]. However, the effect on antibody formation overall is modest, as antibodies form even in TLR9 knockout mice [37]. IgG2a formation (the Th1-dependent murine equivalent of human IgG1) is dependent on intrinsic MyD88 in B cells [37,39]. Antibodies that form against FIX upon AAV gene transfer in mouse and dog models, however, primarily reflect class-switch to Th2-dependent immunoglobulin (IgG1 in the mouse, equivalent to human IgG4).

3.3. Role of the underlying F9 mutation

The risk of antibody formation against FIX is substantially elevated for F9 null mutations (such as a gene deletion) and lowest for missense mutations with circulating antigen [28,40–42]. In transgenic mouse models, endogenous expression of part of FIX antigen was more protective of CD8$^+$ T cell than of antibody responses [28]. Even lack of endogenous expression of the dominant CD8$^+$ T cell epitope did not result in CD8$^+$ T cell activation against FIX as long as the CD4$^+$ T cell epitope was not deleted. Using hemophilia B dogs with a F9 missense mutation (that results in a lack of circulating antigen) as a model, the vector dose per site of injection was identified as another factor that determines the risk of antibody formation [41]. Using a serotype with higher transduction efficiency in muscle (AAV1 instead of AAV2), FIX inhibitors occurred at lower doses [43]. These observations may reflect transduction of APCs, thus resulting in transgene expression in APCs, as has been suggested to occur with AAV1, and to lesser extent with AAV8 [44,45]. However, these outcomes may also be the result of increased loading of dendritic cells (DCs) and other APCs with antigen due to higher local antigen concentrations, and thus increased presentation to T cells. DCs sample antigen in tissues and are critical for initiation of primary immune responses. Based on all these considerations, enrollment of patients into the first gene therapy trial on AAV2-FIX gene transfer to skeletal muscle in the late 1990s was limited to patients with F9 missense mutations, and the vector dose per site was also limited [9,19].

4. Preventing immune responses – from global immune suppression to antigen-specific tolerance approaches

4.1. Use of general immune suppression

Both in canine and murine models of hemophilia B, a transient (6-week) immune suppression protocol (using cyclophosphamide) around the time of intramuscular vector administration blocked antibody

formation against FIX long-term [40–42]. Cyclophosphamide is a DNA alkylating agent that is toxic to dividing cells such as proliferating lymphocytes. Similarly, cyclophosphamide was effective in suppressing antibody formation against FIX in hemophilia B dogs that received muscle gene transfer by isolated limb perfusion, an approach that includes drugs that increase vascular permeability but may enhance inflammation in the tissue such as histamine [46]. Anti-FIX formation seemed reduced in a subsequent study that utilized a different vascular delivery technique, termed 'retrograde infusion", to deliver AAV-FIX to skeletal muscle of hemophilic dogs, suggesting that not only the target tissue but also the route of administration and delivery technique matter (e.g. this method does not involve drugs to increase vascular permeability) [47]. It is encouraging that global immune suppression, although an unattractive solution to solving the risk of immune responses, can be effective long-term when given only transiently. Potential mechanisms include lack of activation signals (which occur early after vector administration and may be derived from tissue damage or innate immunity against the vector) after immune suppression is stopped and/or induction of a lasting transgene product-specific Treg response.

Nonetheless, more targeted approaches are being developed. Evidence has been provided that $CD4^+CD25^+FoxP3^+$ regulatory T cells (Treg) can suppress immune responses to transgene product and to capsid in skeletal muscle [48,49]. $FoxP3^+$ Treg suppress using a variety of cell contact-dependent and -independent mechanisms. For example, *ex vivo* expanded autologous polyclonal Treg can generate an immune regulatory environment upon transplantation, in which activation of effector T cells (Teff) is limited and endogenous antigen-specific Treg are induced [49]. Future protocols may utilize engieneered antigen-specific Treg, which should be effective at lower cell doses [50,51].

4.2. Regulatory T cell (Treg) induction to promote tolerance

Co-administration of the immune suppressive drug rapamycin and antigen results in deletion of Teff (via activation-induced cell death) and induction of $FoxP3^+$ Treg, thereby promoting immune tolerance to the antigen. Induced Treg are more resistant to blockage of the mTOR pathway by rapamycin because they utilize alternative signaling and metabolic pathways (that are independent of mTOR) [52]. Shifting the balance from Teff to Treg responses can be further enhanced by addition of other molecules such as the cytokines IL-10 or Flt3L [53,54]. Such protocols were successful in prevention and reversal of antibody responses against FIX in muscle gene transfer in hemophilia B mice with F9 gene deletion [54,55]. FLt3L does not aid in the induction of Treg but expands existing Treg *in vivo* indirectly through DC expansion [56]. Interestingly, in the presence of rapamycin, FLt3L enhances Treg induction through selective expansion of plasmacytoid DCs (pDCs), when dosed properly [53]. Although FL3L-Flt3 signaling occurs through the mTOR pathway, pDC have an up-regulated mTOR pathway and are therefore more resistant to rapamycin due to a miRNA expression pattern that is distinct from other DCs [57]. It is believed that IDO enzymatic activity in pDCs favors Treg induction [58]. In order to limit global immune suppressive effects of rapamycin, nanoparticle technologies have been developed to package rapamycin. When co-packaged or co-administered with antigen, tolerance may be induced [59,60]. Approaches to antigen-specific tolerance induction without the use of immune suppressive drugs include oral tolerance induction, as shown by a recent proof-of-concept study [61]. Oral tolerance is mediated by the induction of multiple subsets of Treg, including $FoxP3^+$ Treg and $CD4^+CD25^-LAP^+$ Treg that suppress via a TGF-β dependent mechanism and also express the immune suppressive cytokine IL-10 [62–66].

5. Immune tolerance induction to the transgene product by liver gene transfer

5.1. Hepatic gene transfer as a tolerogenic route

In contrast to the experience in skeletal muscle, immune tolerance to FIX can be induced by hepatic AAV gene transfer in multiple strains of hemophilic mice with F9 gene deletion using different expression cassettes and serotypes [67]. Even after challenge with FIX in a strong adjuvant, the mice remained tolerant. Using AAV or other vector systems, a large number of studies have since documented immune tolerance induction to different transgene products by liver-directed gene transfer [68–70]. Tolerance induction to FIX was also successful in dogs with a F9 null mutation, and supporting evidence has been reported in non-human primate studies [71–74]. Therefore, patients enrolled in clinical trials on hepatic AAV-FIX gene transfer have not been limited to those with missense mutations. Despite B and T cell responses to AAV capsid, no immune responses to FIX (or FVIII) have thus far been observed in multiple trials, in which vectors of various serotypes were injected via hepatic artery or peripheral vein for expression of the transgene from a hepatocyte-specific promoter [7,8,75–78].

Expression of an antigen in the hepatocytes takes advantage of the hepatic environment, which favors immune regulation, thus facilitating engraftment of liver transplants but also permitting persistent viral infections such as in viral hepatitis. Specialized cell types such as liver sinusoidal endothelial cells (LSEC) and resident macrophages (Kupffer cells) perform tolerogenic antigen presentation to T cells and may express immune suppressive cytokines, creating an environment that favors Treg induction and suppresses inflammation [79]. Liver draining lymph nodes are major sites of MHC II presentation of transgene product to $CD4^+$ T cells, which requires both DCs and macrophages [80]. Induced Treg quite rapidly disseminate, thus helping to establish tolerance systemically. In addition to this extra-thymic induction of Treg, thymic-derived Treg are also observed by ~2 months after gene transfer for secreted transgene products such as FIX [80,81]. Innate immune responses to AAV in the liver are TLR9 dependent and occur rapidly but are also weak compared to other viral vectors and subside within < 24 h [38]. Adequate levels of transgene expression from hepatocytes (as determined by vector dose, promoter strength, and codon-usage, among other factors) induce immune tolerance through a combination of mechanisms [67,82–84]. However, distribution of transgene expression in the liver may also play a role in addition to expression levels.

5.2. Mechanism of hepatic tolerance induction

Hepatocyte-derived transgene expression causes apoptotic cells death of Teff, some of which reflecting Fas-FasL mediated cell death [67,85,86]. Evidence suggests that hepatocytes transduced with AAV upregulate FasL, and Fas-deficient mice could not be tolerized to FIX by liver gene transfer [67,86]. Expression of a secreted transgene product may tap into central in addition to peripheral tolerance mechanisms. Studies in T cell receptor transgenic mice provided evidence for deletion of transgene product-specific $CD4^+$ T cells and induction of T cell anergy [85]. Moreover, the remaining population of transgene product-specific $CD4^+$ T cells is further suppressed by the TGF-β dependent induction of $FoxP3^+$ Treg [81,85]. Induced Treg suppress antibody and $CD8^+$ T cell responses against the transgene product [87–89]. Higher levels in hepatic transgene expression result in more $FoxP3^+$ Treg induction [82,83]. IL-10 expressed by Treg and by Kupffer cells contributes to suppression of the T cell response but is dispensable for suppression of antibody formation (which is in contrast to oral tolerance induction) [63,81,90]. Viral infections of the liver may also induce IL-10 producing type 1 regulatory T cells (Tr1 cells) [91]. These mechanisms are summarized in Fig. 2.

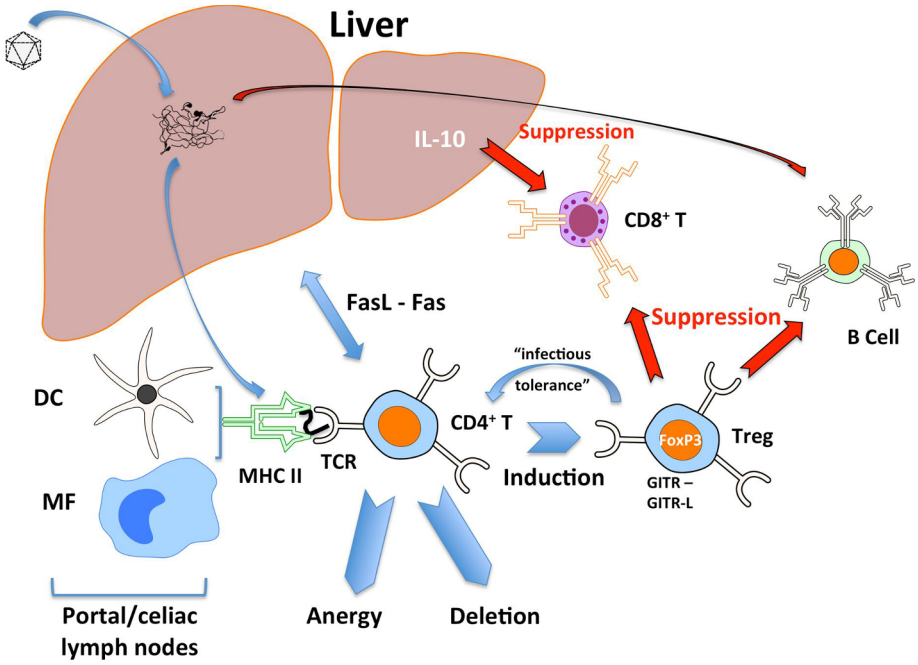

Fig. 2. Mechanisms of antigen presentation and immune tolerance induction to the transgene product by hepatic AAV gene transfer. DC: dendritic cell; MF: macrophage; IL_01: interleukin 10; Fas: death receptor leading to programmed cell death; FasL: Fas ligand.

5.3. Immune modulatory and immune tolerance induction (ITI) gene therapy

In addition to providing therapy while avoiding immune responses to the transgene product, hepatic gene transfer offers additional opportunities as an immune modulatory therapy. One application is the reversal of humoral immune responses that formed in protein therapy. For example, AAV and lentiviral gene transfer to the liver reversed pathogenic antibodies against FIX in hemophilia B mice [83,92]. Gene transfer eliminated life-threatening anaphylactic reactions, even when intravenous FIX protein injections were subsequently resumed. Interestingly, increased levels of transgene expression not only increased Treg induction but also direct suppression of FIX-specific memory B cells. Reversal of inhibitors was also achieved in large animal studies, namely in hemophilia A and B dogs [71,93]. In all these cases, reversal of inhibitor formation resulted in sustained correction of coagulation via stable hepatic gene transfer. For FIX inhibitor reversal, a hyperactive variant was expressed (FIX-Padua), which differs in a single amino acid residue compared to wild-type FIX (which does not appear to increase immunogenicity) [94]. Thus, hepatic AAV gene therapy may be an alternative to current ITI in hemophilia, which is based on frequent high-dose intravenous factor injection and thus very expensive.

In contrast to the canine model, reversal in hemophilia A mice by hepatic AAV gene transfer has been challenging [95]. This may reflect differences in generation of long-lived plasma cells between the two species in response to FVIII, which may be harder to suppress. However, preventive tolerance induction has been accomplished in the mice using optimized expression cassettes [84,95,96]. High levels of FVIII expression in hepatocytes has some potential for induction of a cellular stress response, which however does not appear to increase immunogenicity [96,97]. An alternative method to reverse FVIII inhibitors is the combination of B cell depletion with monoclonal anti-CD20 and rapamycin, which work synergistically on the B and T cell compartments [98]. This combination protocol of rituximab and rapamycin has also been effective in preventing antibody formation in enzyme or gene replacement therapy for Pompe disease (see below) when initiated prior to antigen exposure [99–101].

Induced Treg can enforce immune tolerance at extra-hepatic sites, so that immune tolerance from hepatic AAV gene transfer becomes dominant. As a result, immune responses to intravenously injected protein or to transgene expression in other tissues such as CNS or skeletal muscle are prevented [83,89,102–104]. Protocols to perform gene transfer to other organs simultaneously with or subsequent to hepatic gene transfer have been successful in pre-clinical studies [29,89,102–104]. This strategy represents one potential solution to prevent immune responses to acid α-glucosidase (GAA) in systemic gene therapy for glycogen storage disorder II (Pompe disease). Other opportunities for this tolerogenic gene therapy lie in the treatment of autoimmune disease (at least those with know target antigens) and allergies [105,106]. For human translation, the approach should benefit from the development of AAV capsids with superior transduction of human hepatocytes [107].

5.4. Limitations to tolerance induction by hepatic gene transfer

One should also caution that suboptimal hepatic gene transfer may fail to induce tolerance. For example, using secreted ovalbumin as a model transgene product, we found that low vector doses can activate $CD8^+$ T cells that are functionally not fully competent, expressing negative checkpoint regulators such as PD-1 and other markers also found in exhausted T cells [108]. These T cells are initially kept from mounting a destructive response through in situ mechanisms in the hepatic environment such as the PD-1/PD-L1 pathway. However, the T cells may down-regulate PD-1, up-regulate cytokine expression, and acquire functionally after 2–3 months, thus eliminating transgene expressing hepatocytes. These results are reminiscent of loss FIX expressing hepatocytes 2–3 months after AAV2 gene transfer to a specific strain of hemophilia B mice (C3H/HeJ with F9 gene deletion) that was not observed when using more potent vectors [28]. Tolerance induction to FIX has also not been successful in some of hepatic AAV-transduced non-human primates. In this subset of animals, antibody formation was reversed using immune suppression, which consisted of B cell depletion with anti-CD20 and administration of the calcineurin inhibitor cyclosporine (thus inhibiting T cell receptor signaling), followed by cyclophosphamide [77,109]. Here, antibody formation rather than cytotoxic T cell responses had occurred, so that systemic FIX expression re-appeared after elimination of the antibodies.

6. Clinical challenges to avoid immune responses to AAV-encoded transgene products

6.1. $CD8^+$ T cell responses to transgene products observed in clinical trials

Pre-clinical studies suggest that the possibility of antibody formation against secreted transgene products following muscle gene transfer is not only a concern for treatment of genetic disease but also for systemic delivery of antibodies to protect from viral pathogens such as HIV [110]. A potentially greater challenge arises for systemic delivery of vector to multiple organs, for example in muscular dystrophy or lysosomal storage disorders. Thus far, antibody formation against AAV transgene products has not been described in human patients. However, there have been at least 2 examples of $CD8^+$ T cell responses to transgene products after AAV muscle gene transfer. One example is Duchenne's muscular dystrophy. Here, pre-existing $CD8^+$ T cell immunity has been reported in some patients, possibly resulting from intermittent expression of dystrophin antigen by revertant fibers in the context of tissue inflammation [6]. These investigations document the potential for T cell immunity to self- and non-self dystrophin epitopes, as well as effectiveness of immune suppression [111]. Another example is α_1-antitrypsin deficiency. AAV1 gene transfer activated $CD8^+$ T cells in at least one, which was surprising, given that the subject had a missense mutation [5]. Interestingly, the response was not directed against an epitope that spans the mutation but rather against a mismatch between the therapeutic gene and a polymorphism in the human population. This issue has been controversial in FVIII replacement therapy for hemophilia A. For example, African-American patients have an elevated risk for developing inhibitory antibodies against FVIII in traditional protein therapy, which one study attributed to polymorphic differences between the therapeutic recombinant protein and these patients [112]. However, a subsequent investigation found no evidence for T cell reactivity against such epitopes [113].

6.2. Critical questions for the future of clinical gene therapy

Transient immune suppression with steroids or B cell depletion with anti-CD20 combined with rapamycin are among the approaches that have seen first clinical use to prevent unwanted immune responses in gene therapy for muscular dystrophy and storage disorders [77,111,114]. Going forward, critical questions for the new protocols will be: is immune suppression needed? How long should immune suppression be continued, and will tolerance be maintained after the regimen is discontinued? It is encouraging that pre-clinical studies with FIX, for example, have suggested that transient immune suppression can result in long-term unresponsiveness to the transgene product [40,42,55]. Can we replace immune suppression with protocols for antigen-specific tolerance induction? When using tolerogenic routes of vector administration, such as the liver, will these pre-clinical concepts translate to humans? Studies in non-human primates have given mixed results, some supporting tolerance induction, while antibody formation was observed in others. On the other hand, tolerance induction by hepatic AAV gene transfer has been quite successful in hemophilia A and B dogs with pre-existing immunity [71,93]. Translation of this approach will in part also depend on solving the problem of T cell responses to the viral capsid [115].

In summary, detailed studies on FIX expressing upon AAV gene transfer in various animal models have provided the field with detailed knowledge on the role of vector design and dose, underlying mutation of the deficient host gene, target organ, route of administration, and effectiveness of immune suppression, among others. Moreover, various immune tolerance protocols have emerged, such as hepatic gene transfer or combination of gene therapy and drug- or cell therapy. These concepts will be useful to avoid unwanted immune responses to other transgene products.

Author contributions

RWH wrote the manuscript.

Conflict of Interest

RWH received royalty payments from Spark Therapeutics for license of AAV gene transfer technology and serves on a scientific advisory board for Applied Genetic Technologies Corporation (AGTC).

Acknowledgments

This work was supported by NIH grants R01 AI51390 and R01 HL131093.

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