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Update on Clinical Gene Therapy for Hemophilia

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Abstract

In contrast to other diverse therapies for the X-linked bleeding disorder hemophilia that are currently in clinical development, gene therapy holds the promise of a lasting cure with a single drug administration. Near to complete correction of hemophilia A (factor VIII deficiency) and hemophilia B (factor IX deficiency) has now been achieved in patients by hepatic in vivo gene transfer. Adeno-associated viral (AAV) vectors with different viral capsids and engineered to express high-levels and in some cases hyperactive coagulation factors were employed. Patient data support that sustained endogenous production of clotting factor as a result of gene therapy eliminates the need for infusion of coagulation factors (or alternative drugs that promote coagulation), and may therefore ultimately also reduce treatment costs. However, mild liver toxicities have been observed in some patients receiving high vector doses. In some but not all instances, the toxicities correlated with a T cell response directed against the viral capsid, prompting use of immune suppression. In addition, not all patients can be treated because of pre-existing immunity to viral capsids. Nonetheless, studies in animal models of hemophilia suggest that the approach can also be used for immune tolerance induction, to prevent or eliminate inhibitory antibodies against coagulation factors. These can form in traditional protein replacement therapy and represent a major complication of treatment. The current review provides a summary and update on advances in clinical gene therapies for hemophilia and its continued development.

Introduction

Hemophilia is an X-linked monogenic coagulation disorder resulting from a deficiency in coagulation factors in the intrinsic coagulation cascade.[1,2] Hemophilia A, the more prevalent form of hemophilia occurs in 1 in 5,000 live male births and is caused by a mutation in the gene coding for factor VIII (FVIII) resulting in the loss of functional FVIII protein. FVIII is a critical co-factor for the serine protease factor IX (FIX), which is deficient in hemophilia B patients. Both FVIII and FIX are naturally synthesized in the liver, FVIII in liver sinusoidal endothelial cells (LSEC) and FIX in hepatocytes. It is estimated that there is a total of 20,000 hemophilia patients in the US, with hemophilia A being about six times more common than hemophilia B. Clinically, both hemophilia A and B patients are segregated into three groups based on residual coagulation factor activity: severe (<1%), moderate (1-5%), and mild (5-40%). Untreated severe hemophilia patients are at risk for either morbidity or mortality from spontaneous or trauma induced bleeds. The most common form of morbidity is hemophilic arthropathy resulting from recurrent bleeds into the joints. Moderate hemophilia patients have a significant reduction in spontaneous bleeds, but are still at risk from trauma induced bleeds, while mild hemophilia patients may appear phenotypically normal and never show signs of uncontrolled bleeds unless undergoing severe trauma or surgery.

Current recommended therapy for hemophilia is prophylactic administration of exogenous coagulation factors derived from pooled plasma or recombinant protein. The short biological half-lives of FVIII and FIX proteins require frequent infusions (2-3 times per week) to maintain trough levels above 1%, the minimally effective level to significantly reduce the incidence of spontaneous bleeds. A major complication of factor replacement therapy is the formation of anti-drug antibodies, termed inhibitors.[3] Inhibitors form in approximately 25-30% of hemophilia A patients and less frequently in 3-5% hemophilia B patients. Clinically, patients with an inhibitor titer above 5 Bethesda units (one Bethesda unit is defined as the amount of antibody that reduces factor activity by 50%) are no longer responsive to factor replacement and require

treatment with bypassing agents to maintain hemostasis. Traditional bypassing agents, such as activated prothrombin complex concentrate and recombinant activated factor VII are generally expensive, have short biological half-lives, and are not as effective as FVIII or FIX in long-term hemostasis. Alternatively, inhibitor patients can be placed on an immune tolerance induction (ITI) protocol requiring frequent infusions of super physiological levels of coagulation factor until inhibitors are reduced or eliminated and patients can resume factor replacement therapy.[4,5] While effective in approximately two-thirds of hemophilia A patients with inhibitors, ITI often has to be discontinued in hemophilia B patients due to the development of anaphylaxis and nephrotic syndrome.[6] ITI therapy is expensive, places a significant burden on the patient, and the long-duration of therapy increases the risk for bleeds.[7] Considering the high life time costs, frequencies of infusions, and potential health burden there is a need for alternative cost-effective therapies with reduced risk and improved efficacy for hemophilia.

Rationale for gene therapy for hemophilia

Gene therapy provides a functional copy of the disease-causing gene that is either absent or expressed as a nonfunctional protein thus, it can be highly effective in treating monogenic disease, such as hemophilia. The initial barrier of inefficient delivery of the therapeutic genetic payload into target cells and tissues was circumvented through the adoption of viral vectors derived from mammalian viruses, that have naturally evolved to deliver their genetic cargo into cells and tissues. These vectors contain minimal wild-type viral sequences and their pathogenic, replicative, and structural viral genes are replaced with the therapeutic gene cassette. Over the years, hepatic *in vivo* gene transfer using adeno-associated virus (AAV) vectors has shown the best success in pre-clinical and clinical studies, with several clinical studies for both hemophilia A and B enrolling patients for phase III trials.

Hemophilia is well suited for correction by gene therapy because the bleeding phenotype is responsive to a wide range of factor levels and precise regulation is not necessary. Further,

since clotting factor proteins are secreted into the circulation, it is possible to correct the bleeding diathesis with gene delivery to a fraction of total hepatocytes. FVIII and FIX can be synthesized in non-native cells and tissues. For example, even though FVIII is naturally secreted by specialized endothelial such as LSEC and extrahepatic endothelial cells, expression in hepatocytes generates a functional protein that has restored hemostasis in animal models and human patients. And finally, for patients in developing countries with comorbidities and mortalities resulting from inadequate factor supply, gene therapy could provide a significant benefit by providing a continuous source of clotting factor from a single treatment.

Adeno-associated viral (AAV) vectors

AAV vectors are derived from wild-type AAV,[8] a member of the parvovirus family. Wild-type AAV is non-pathogenic, weakly immunogenic, and replication deficient, requiring a helper virus for replication. AAV vectors can deliver a therapeutic transgene cassette up to 5 kb into both dividing and non-dividing cells. However, the majority of AAV vector genomes do not integrate into the host genome and gene delivery into dividing cells results in progressive loss of transgene. AAV serotypes are segregated into clades based on viral capsid homology.[9] The viral capsid governs both tissue tropism and intracellular viral particle trafficking. Engineered AAV capsids generated by random and directed mutagenesis and capsid gene shuffling have been created to enhance human hepatocyte tropism, resist neutralizing antibodies, and evade elimination of transduced hepatocytes by cytotoxic T cells.[10-12] Engineering of the expression cassette continues to be a key component for successful gene therapy. For instance, vector potency can be enhanced through the design of stronger synthetic liver-specific promoters, codon-optimized *F8* and *F9* cDNAs, use of engineered *F8* (B-domain deleted FVIII variants) and *F9* (hyper active FIX-Padua variant R338L) genes.[13-19] At the same time, there is concern that overexpression in particular of FVIII could result in cellular stress and unwanted toxicities.[20-22] Some clinical trials utilize expression cassettes that have been edited to eliminate of immune

stimulatory CpG motifs, which have the potential to enhance $CD8^+$ T cell responses.[14,23] However, since the inverted terminal repeats of the viral genome also contain CpG motifs, their complete elimination from the vector is not possible.

AAV vector liver directed gene therapy in hemophilia animal models induces immune tolerance to FVIII and FIX through induction of factor specific regulatory T cells (Treg).[24-29] There is now strong evidence that AAV and lentiviral (LV) vector liver directed gene therapy can also eliminate established inhibitors.[30] For instance, AAV-F8 and AAV-F9 vector liver gene transfer was an effective ITI in canine hemophilia models.[27,28] Similarly, LV-F9[31] and AAV-F9[26] vector liver gene transfer could eradicate pathogenic inhibitors in hemophilia B mice. While these results are encouraging, translatability to humans will have to be confirmed.

Immunology of AAV gene therapy

Immune recognition by cytotoxic $CD8^+$ T cells or antibody responses to the vector capsid, the transgene product, or both can compromise therapeutic expression of the transgene (Figure 1).[32-34] However, an immune response that is detectable by *in vitro* assays does not always affect transgene expression *in vivo*.[18,35] Humans are naturally infected with wild-type AAV during childhood and thus may develop neutralizing antibodies (NABs) that often cross-react with multiple serotypes and prevent gene transfer with AAV vectors. Similarly, NAB development upon gene transfer prevents patients from receiving repeat administration of the same AAV vector at least in the near future. Activation of capsid-specific $CD8^+$ T cells may result in targeting of transduced hepatocytes, mild and transient transaminitis, and loss in factor levels.[35] Hence, an immune suppression protocol was developed based on steroid drugs that are given in response to increases in transaminases in the blood.[14] Rapid employment is required to preserve transgene expression. Compared to T cell assays, elevation in transaminases allows for more rapid treatment response. While there is evidence for memory $CD8^+$ T cells to capsid,

this response is not fully understood in humans, and loss of transgene expression or hepatotoxicity could not always be correlated with a T cell response.[23,35,36] Therefore, a better understanding of the innate and adaptive immune responses to AAV vectors as well as alternative sources of toxicity is needed to ensure long-term gene expression.[11,37,38]

Vector dose and transgene expression levels have a significant impact on whether an immune response is induced against both the AAV vector and transgene product.[35,38] Importantly, optimal transgene expression in the hepatic environment can lead to immune tolerance to the therapeutic protein antigen, and to date no clinical gene therapy study has reported detecting B or T cell response directed against either FVIII or FIX.[14,23,36,39-41] Initial clinical studies for AAV gene therapy for hemophilia have been conducted in adults >18 years of age, in which patients have had sufficient exposure days without an inhibitor, a common exclusion criterion. Additional rigorous clinical studies will be needed in previously untreated patients, more so for hemophilia A patients where the inhibitor frequency is 25-30%, to determine the relative risk of inhibitor formation following gene transfer. Further, since the expression cassettes for FVIII and FIX are episomal, treating a patient too early, while the liver is still growing, may cause a loss in expression. If a patient would require additional vector infusions, strategies to prevent the formation of neutralizing antibodies against the vector capsid will have to be developed, or a vector with diverse capsid sequence will have to be used for the second administration.

AAV gene therapy clinical trials

AAV vectors have progressed into clinical studies for both hemophilia A and B. Gene transfer of a scAAV8-co*F9* vector to human hepatocytes in adult hemophilia B patients has provided stable expression of FIX protein now for greater than six years.[40] The clinicaltrials.gov database presently lists nine active clinical trials evaluating different AAV capsid serotypes and *F9* gene sequences for hemophilia B and nine trials to evaluate AAV-*F8* gene therapy for

hemophilia A (**Table 1**) with some overlap as the same vector is being evaluated in both phase I/II and III studies. Although ongoing clinical trials for hemophilia have been recently reviewed elsewhere,[42-48] this review will focus on several notable trials that have recently been published or presented updates at national meetings for the American Society of Hematology and American Society of Cell and Gene Therapy.

Hemophilia A clinical trials

BioMarin initiated the first clinical trial for hemophilia A using liver gene transfer of an AAV5 vector expressing a codon optimized BDD-FVIII-SQ protein, BMN 270.[36] An initial patient treated at a low vector dose had nearly undetectable FVIII levels with little change in FVIII protein usage. A second patient treated at an ~3-fold higher dose had stable FVIII levels between 1-3% normal and discontinued prophylaxis FVIII protein usage. Patients in the highest dose cohort (reported as $6x10^{13}$ vector genomes [vg]/kg) expressed a range of therapeutic FVIII levels with median of 77% and range of 19-164% of normal at 52 weeks, with a significant reduction in recombinant FVIII usage and annual bleeding rate. Most patients treated in the high dose cohort received an extended course of prophylactic prednisolone in response to mild elevations in transaminase observed in participant 3. Participant 4 developed transaminitis following cessation of the initial round of steroid treatment and was placed back on a second round of tapered steroids. Elevation in transaminases did not correlate to a T cell response, prompting the authors to speculate that vector infusion itself combined with innate sensing of viral molecular structures may have caused mild liver inflammation.[36] A step-down dose cohort with six participants received 2/3 of the highest dose. A median expression of 33% of normal at 24 weeks post vector treatment and discontinuation of FVIII protein therapy with no reported bleeds was documented. BioMarin reported at the 2018 World Federation of Hemophilia congress some reduction in the median FVIII levels at 104 weeks, with follow-up studies forthcoming.

BMN 270 was produced using a baculoviral insect cell-based production system. This was the first hemophilia trial to use rapid vector dose escalation (vector dose was raised after the first treated patient failed to show any correction), and prophylactic prednisolone administration. BMN 270 was recently assigned the commercial name of Valoctocogene Roxaparvovec. Two phase III studies with an estimated enrollment of 130 patients total have been initiated with target vector dose of $4x10^{13}$ vg/kg and $6x10^{13}$ vg/kg. Additionally, BioMarin is enrolling by invitation a phase I/II study to evaluate high-dose Valoctocogene Roxaparvovec gene delivery in hemophilia A patients with pre-existing AAV5 capsid antibodies.

Spark Therapeutics has presented preliminary trial data on hemophilia A patients treated with an initial low vector dose in two patients, followed by three patients treated with intermediate dose, and the seven treated with high dose of their Spk8011 vector (representing 2-fold dose escalations between cohorts).[49-51] The reported vector doses ranged from $5x10^{11}$ to $2x10^{12}$ vg/kg. While these numbers indicate usage of substantially lower vector doses compared to the trial with AAV5, one needs to caution that titers were not determined side-by-side and were likely not based on identical protocols. The first two patients at the initial vector expressed 10% and 13% normal levels of FVIII protein and two of the three mid dose treated patients expressed 8% and 12% of normal FVIII protein. According to data released by Spark Therapeutics, higher levels of expression (on average ~30% and up to 49% of normal FVIII activity by 3 months) but also toxicities were observed at the highest vector dose. This trial is notable as it is the first use of capsid that was generated from a capsid library following selection for human hepatocyte tropism and resistance to antibody neutralization.[10]

Hemophilia B clinical trials

Of the many ongoing hemophilia B AAV clinical trials, there are several notable successes. The trial sponsored by University College, London (UCL) and St. Jude Children's Research Hospital for hemophilia B patients using an scAAV2/8-LP1-hFIXco vector was the first long-term

success story for hemophilia gene therapy.[40] The first patient was treated in 2009, making this trial the longest follow up on the safety and stability of FIX protein expression from an AAV vector in the liver. However, several patients had an early decline in FIX levels related to transient transaminitis associated with a capsid T cell response that stabilized following treatment with tapered prednisolone. Last reported FIX levels in the high dose cohort ($2x10^{12}$ vg/kg) of six patients were a mean of 6.1% with a range of 2.89-7.2%. Going forward, the UCL has initiated a new phase I/II trial for hemophilia B using an undisclosed (capsid and transgene) AAV-*F9* vector, FLT180a. As of this review, no data has been released.

Spark Therapeutics has initiated a hemophilia B clinical trial, using an AAV8 capsid with several amino acid substitutions to package a hyperactive FIX-Padua expression cassette.[39] FIX-Padua is a naturally occurring hyperactive FIX variant (R338L) with a reported approximately 8-fold increase in specific activity.[52] Ten patients were treated at a dose of $5x10^{11}$ vg/kg and had a mean FIX activity of 33.7% with a range of 14-81%. Two participants, 7 and 9, had asymptomatic transient transaminitis which was resolved through steroid treatment and only one subject showed moderate loss in FIX activity. Eight of the ten treated patients had stable FIX activity during reported follow-up and no indication of an AAV capsid T cell response. Presently, new patients are being enrolled for a phase III trial.

UniQure has recently published data on their clinical trial for hemophilia B with an AAV5 vector using the same *F9* expression cassette as the UCL and St. Jude Children's Research Hospital trial.[14,23,40] Ten patients were enrolled, and five received a low dose while another five were treated at a 4-fold higher dose. Mean FIX levels in the low dose cohort were 4.4% with a range of 1.3-6.8%. Mean FIX in the higher dose cohort were 6.9% with a range of 3.1-12.7%. However, it appears that both hemophilia B trials by UCL and Spark utilized lower vector doses to achieve similar or higher efficacy. Similar to the BioMarin trial several patients displayed transient transaminitis without indication of a capsid T cell response. Given the high vector doses used, it may be of interest to examine other potential causes for hepatotoxicity in these

vector preparations.[53] A new dose confirmation trial using the FIX-Padua variant at the high-dose (reported as $2x10^{13}$ vg/kg) is planned and expected to enroll three hemophilia B patients and depending on outcomes will proceed to a phase III study.

There are several hemophilia B AAV liver gene therapy trials that have been completed or terminated on the clinicaltrials.gov database, primarily due to lack of efficacy. However, most of these trials have never published patient outcomes in a peer reviewed journal, so there is limited publicly available data.[35,54] A common reporting of most of these studies is transient transaminitis, in either the presence or absence of detectable capsid-specific $CD8^+$ T cells.

Challenges to clinical implementation of AAV gene therapy

As discussed above, there are a number of immunological hurdles to AAV-mediated gene therapy for hemophilia. Additional challenges are summarized in the following.

Production scale-up of AAV vectors

For widespread therapeutic use, suitable scalable production methods will be needed to produce sufficient quantities of clinical-grade AAV vectors. Either transfection of mammalian cells, such as HEK293 cells, or baculoviral production systems in insect cells have been used to manufacture AAV vectors in all hemophilia clinical trials to date.[18] While further improvements are being made to the baculoviral system, a vaccinia virus-based platform has more recently been developed for scale up in mammalian cells.[55,56] Permanent production cell lines for AAV are also under development. The potential role of the production system in immune responses and toxicities remains to be defined.

Non-immune toxicities related to gene transfer

The risk of insertional mutagenesis following AAV mediated gene transfer is low since the majority of vector genomes persist episomally. Even though natural infection with AAV is not

linked to cancer, deep sequencing studies show that integration of the AAV genome can occur in the liver.[57,58] Additionally, an increased incidence of hepatocellular carcinoma (HCC) has been reported in the mucopolysaccharidoses type VII (MPSVII) mouse model following perinatal gene transfer of AAV potentially through integration and disruption of an imprinted region rich in miRNAs and snoRNAs on mouse chromosome 12.[59] Subsequent studies in other murine models have failed to recapitulate this finding. However, an extensive study in mice revealed vector design can modulate the relative risk of HCC development.[60] Collectively, the available data in various animal models suggest that AAV has a relatively low risk of tumorigenesis,[61] though AAV integrations appear to be present in some human HCC.[62] There are several recent studies demonstrating links with HCC and wild-type AAV.[63,64] A debate whether this extends to AAV vectors is ongoing.[65-67] Though there is substantially more evidence supporting no risk of insertional mutagenesis in animal models and AAV treated hemophilia patients, greater numbers of treated patients are still needed to completely rule out this as a potential risk.[18]

Another potential toxicity, related specifically to AAV gene therapy for hemophilia A, has been described by recent research. This work demonstrated that over-expression of FVIII in hepatocytes may induce cellular stress,[20,21] due to accumulation of misfolded FVIII protein in the ER. Since hepatocytes do not naturally express FVIII protein nor the closely associated vWF protein, the risk for ER induced stress in hepatocytes is a potential concern. However, it not clear that an ER stress response to FVIII occurred in AAV treated hemophilia A patients, as discussed by the investigators.[36] Nonetheless, pre-clinical studies have focused on engineering FVIII proteins that are more efficiently secreted from hepatocytes[15,68-72] including the FVIII variant, FVIII-V3, which is now being evaluated in clinical trial (Table 1). In addition to ER stress, it was speculated that the mild liver toxicities observed in hemophilia A patients may be related to viral particle trafficking, uncoating, and vector DNA induction of the DNA damage response.[36]

Evolving approaches for hemophilia gene therapy

Gene editing and targeted integration

Gene editing is a molecular technique that can correct the endogenous genetic defect or direct the integration site of a therapeutic gene through nuclease targeted double stand DNA breaks and homology directed repair (HDR). Zinc-finger nucleases (ZFN), transcription activator-like effector-based nucleases (TALENS) and the clustered regularly interspaced short palindromic repeats-Cas system (CRISPR-Cas) systems were developed to induce a double strand break in DNA, vastly increasing the frequency of HDR and editing, or for gene disruption.[73,74] In terms of hemophilia therapeutics, gene editing approaches are being investigated to correct endogenous *F9* mutations or insert *F9* into a defined locus such as safe harbor sites or more recently the albumin locus.[75] Clinical application may be limited by off-target cutting and higher than anticipated frequency of integrations with the non-homologous end joining (NHEJ) pathway. Sangamo Therapeutics is currently recruiting patients utilizing AAV6 to deliver ZFNs and a promoterless *F9* transgene, targeting the albumin locus, to treat hemophilia B. However, at the time of this review there has been no data released. Alternatively, it was shown that an AAV cassette with homology arms is sufficient for targeted integration independent of DSB in mice resulting in FIX levels in the range 7-20% normal, thus avoiding the need for co-delivery of an endonuclease.[76]

Combining gene and immune modulatory therapies

While hepatic gene transfer has the potential to induce immune tolerance, adjunct immune modulation may further enforce tolerance to the FVIII of FIX transgene products. This may be of particular relevance for the more immunogenic FVIII molecule. A number of such combination therapies have been described. The mTOR inhibitor rapamycin has received particular attention as it causes activated induced cell death following signaling through the T cell receptor (TCR) in $CD4^+$ T effector cells, while sparing Treg. In murine models, a one-month regimen based on

administration of rapamycin and FVIII or FIX antigen (in some cases combined with a second drug with synergistic effects) has been successful in promoting tolerance in murine models of hemophilia in the context of protein or gene therapy.[77-79] Nanoparticle delivery of rapamycin is effective at suppressing AAV cellular and humoral immunity.[80] Transient B cell depletion by monoclonal antibody therapy (using anti-CD20) reduced the risk of inhibitor formation in the context of AAV gene therapy[81] and when combined with rapamycin enhanced reversal of FVIII inhibitors in hemophilia A mice.[82] A similar protocol is in clinical development to prevent anti-drug antibodies in other enzyme replacement therapies and to suppress NAB formation against AAV vectors, thus improving the chances for successful re-administration of vector.[83] Of note, there are also non-immune suppressive methods to promote vector administration in an individual with pre-existing NAB to AAV, such as co-administration of decoy capsids ("empty" capsids that lack vector genomes) in excess to saturate NAB binding sites.[84]

Conclusions

Given the exciting breakthroughs with new hemophilia therapies there is debate regarding the benefits of gene therapy considering recently approved enhanced half-life (EHL) factors and other biologics. However, EHL products thus far have failed to show a substantial gain in half-life for FVIII. Biologics that target coagulation pathway inhibitory proteins and a bispecific antibody FVIII mimetic show much promise (but have also been associated with thrombosis in clinical evaluation, perhaps due to a lack of functional regulation that is inherent in FVIII and FIX products).[48,85] Alternative gene therapy approaches targeting hematopoietic stem cells with lineage restricted expression in CD68 monocytes[72] and platelets[86] are being investigated. Targeting expression to platelets may avoid neutralization of FVIII by inhibitors, and also has potential for immune tolerance induction. However, these approaches may require preconditioning of bone marrow at least with a non-myeloablative regimen and transient immune suppression.

Two separate AAV-based gene therapy phase I/II clinical trials for hemophilia A have now reported stable FVIII levels following a single vector dose, demonstrating that gene therapy is the most effective investigational treatment for adult hemophilia A patients. In hemophilia B, even though EHL FIX products have benefited from a greater improvement in half-life, multiple phase I/II gene therapy clinical trials have now reported stable therapeutic FIX expression following a single treatment using an AAV vector. Given the robustness of expression thus far, up to six years, it is likely that AAV gene therapy for hemophilia B will be a highly attractive therapeutic option for patients following the anticipated approval by the FDA. While the clinical data supporting gene therapy for hemophilia has been extremely positive, the enrolled subjects have a preselection bias, and thus some caution is advised in extending the benefits of gene therapy to the general hemophilia patient population. It is unclear how effective AAV gene therapy will be in young children with less than 50 exposure days in terms of the risk of inhibitor and potential loss of factor expression over time due to dilution of AAV transduced hepatocytes during liver growth. Integrating lentiviral vectors (LV) may provide an alternative vector platform for treating children with hemophilia with *in vivo* liver gene delivery of immune stealth and tolerogenic LV vectors to hepatocytes[87,88] and LSEC.[89] In children and adults with either high risk mutations for inhibitors, history of inhibitor, or presently with inhibitor, it is unclear if the robust AAV mediated liver tolerance and ITI observed in hemophilia animal models will translate in humans. Therefore, until such studies have been conducted, there is also a need for the continued development of improved factor and bypassing agent products. Nonetheless, with continued growth and success, AAV gene therapy may replace enzyme replacement therapy as a potential curative therapy for hemophilia.

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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). GQP and DMM have no relevant conflict.

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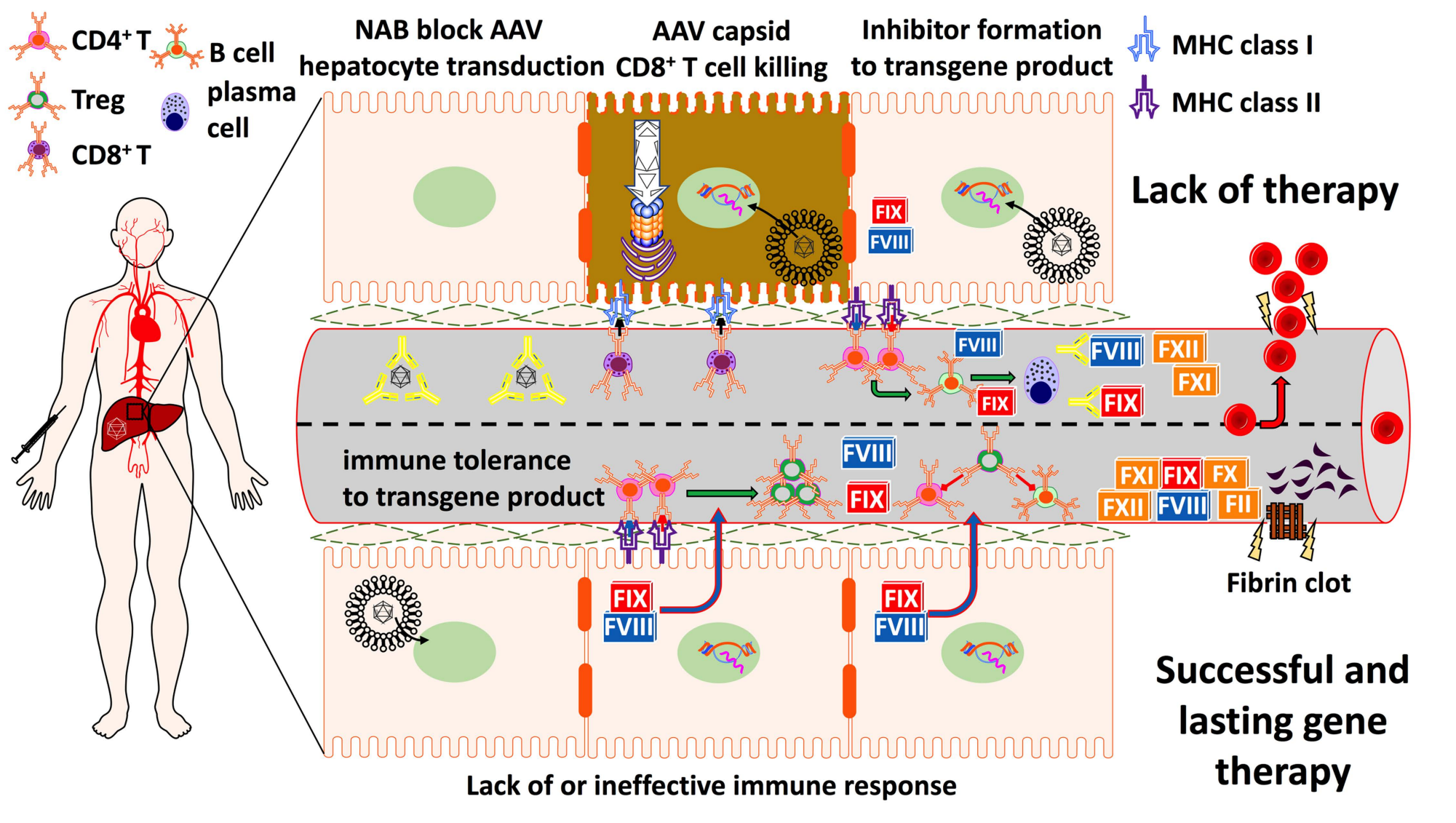
Hemophilia A active gene therapy clinical trials			
NCT Number	**Status**	**Interventions**	**Sponsor/Collaborators**
NCT03370172	Recruiting	BAX 888, AAV8-co-BDD-*F8*	Baxalta now part of Shire|Shire
NCT02576795	Active, not recruiting	BMN 270, AAV5-co-BDD-*F8*	BioMarin Pharmaceutical
NCT03370913	Recruiting	Valoctocogene Roxaparvovec (BMN 270)	BioMarin Pharmaceutical
NCT03392974	Recruiting	Valoctocogene Roxaparvovec (BMN 270)	BioMarin Pharmaceutical
NCT03520712	Enrolling by invitation	Valoctocogene Roxaparvovec (BMN 270)	BioMarin Pharmaceutical
NCT03061201	Recruiting	SB-525, AAV6-co-BDD-*F8*	Sangamo Therapeutics
NCT03003533	Recruiting	SPK-8011, LK03-co-BDD-*F8*	Spark Therapeutics
NCT03432520	Not yet recruiting	SPK-8011, LK03-co-BDD-*F8*	Spark Therapeutics
NCT03001830	Recruiting	AAV2/8-HLP-FVIII-V3	University College, London|Medical Research Council
NCT03217032	Not yet recruiting	YUVA-GT-F801- Lentiviral vector *F8* HSC	Shenzhen Geno-Immune Medical Institute
Hemophilia B active gene therapy clinical trials			
NCT Number	**Status**	**Interventions**	**Sponsor/Collaborators**
NCT03217032	Not yet recruiting	YUVA-GT-F901, Lentiviral vector *F9* HSC	Shenzhen Geno-Immune Medical Institute
NCT01687608	Active, not recruiting	AskBio009, AAV8-co*F9*-Padua	Baxalta now part of Shire|Shire
NCT02695160	Recruiting	SB-FIX, AAV6-ZFN Alb locus and AAV6-*F9* donor template	Sangamo Therapeutics
NCT00515710	Active, not recruiting	AAV2-FIX16	Spark Therapeutics|Children's Hospital of Philadelphia|The Hemophilia Center of Western Pennsylvania|Royal Prince Alfred Hospital, Sydney, Australia
NCT02484092	Active, not recruiting	SPK-9001, mutant AAV8-co*F9*-Padua	Spark Therapeutics|Pfizer
NCT03307980	Recruiting	SPK-9001, mutant AAV8-co*F9*-Padua	Spark Therapeutics|Pfizer
NCT00979238	Active, not recruiting	scAAV2/8-LP1-hFIXco	St. Jude Children's Research Hospital|National Heart, Lung, and Blood Institute (NHLBI)|Hemophilia of Georgia, Inc.|Children's Hospital of Philadelphia
NCT02396342	Active, not recruiting	AAV5-hFIX (AMT-060)	UniQure Biopharma B.V.
NCT03489291	Recruiting	AAV5-hFIXco-Padua (AMT-061)	UniQure Biopharma B.V.
NCT03369444	Recruiting	FLT180a	University College, London

Table 1. Summary of active gene therapy clinical trials for hemophilia registered on clinicaltrials.gov.

Figure Legend:

Figure 1. Summary of immunological challenges and successes of liver-directed AAV gene therapy for hemophilia in humans. Depicted above is a representation of hepatocytes and liver sinusoidal endothelial cells (LSEC) lined blood vessel within the liver. Following intravenous delivery of an AAV vector viral particles enter the hepatocyte, escape the endosome, and deliver their genetic payload to the nucleus. Pre-clinical animal models show that FVIII or FIX expression in the liver induces immunological tolerance through conversion of effector $CD4^+$ T cells into regulatory T cells (Treg). Tregs suppress activation of both B and T cell responses directed against FVIII or FIX. Pre-existing neutralizing antibodies (NAB) to the AAV capsid can block hepatocyte gene delivery and prevent therapeutic correction of the bleeding diathesis. Upon successful gene delivery to hepatocytes, primary or recall immune responses may lead to activation of capsid specific cytotoxic $CD8^+$ T cells and targeted elimination of hepatocytes presenting AAV capsid epitopes on MHC class I. Pre-clinical animal models of severe hemophilia show that suboptimal gene delivery may fail to induce tolerance to the transgene product, because a threshold level of FVIII or FIX expression is needed for successful activation of Treg. In the absence of Treg, effector $CD4^+$ T cells may become activated upon recognition of FVIII or FIX epitopes presented on MHC class II, which is primarily expressed on professional antigen presenting cells (APC). The resulting T help promotes B cell maturation, leading to the production of high affinity class switched antibodies and terminal differentiation into antibody secreting plasma cells.


CD4+ T
B cell
Treg
plasma cell
CD8+ T
NAB block AAV hepatocyte transduction
AAV capsid CD8+ T cell killing
Inhibitor formation to transgene product
MHC class I
MHC class II
Lack of therapy
FIX
FVIII
FXII
FXI
immune tolerance to transgene product
FX
FII
Fibrin clot
Successful and lasting gene therapy
Lack of or ineffective immune response

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