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SPECIAL REPORT



New technologies in gene therapy for inducing immune tolerance in hemophilia A

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ABSTRACT

Introduction: Conventional hemophilia treatment is based on repeated infusion of the missing clotting factor. This therapy is lifelong, expensive and can result in the formation of neutralizing antibodies, thus causing failure of the treatment and requiring higher doses of the replacement drug.

Areas covered: Gene and cell therapies offer the advantage of providing a definitive and long-lasting correction of the mutated gene, promoting its physiological expression and preventing neutralizing antibody development. This review focuses on the most recent approaches that have been shown to prevent and even eradicate immune response toward the replaced factor.

Expert commentary: Despite the encouraging data demonstrated by ongoing clinical trials and pre-clinical studies, more extensive investigations are necessary to establish the long-term safety and efficacy of gene therapy treatments in maintaining immune tolerance.

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Hemophilia; gene and cell therapy; immune tolerance; AAV; lentiviral vectors; genome editing

1. Inhibitor formation in hemophilia A

The hereditary coagulation disorders caused by the absence of clotting factor VIII (FVIII) or IX (FIX) are known as Hemophilia A (HA) and Hemophilia B (HB), respectively. Both genes for FVIII and FIX in humans are located on the X chromosome, and the incidence is higher for HA (1:5000 male births) than HB (1:30,000 male births) [1]. The classical treatment is based on the recurrent infusion of either plasma-derived (pd) or recombinant (r) clotting factors for maintaining hemostasis. Both drug preparations have reached a high level of quality for safety and efficacy, even if there is still an open debate on which is better [2]. Despite the therapeutic improvements introduced by recent products, alloantibodies against the missing factor, called inhibitors, develop in 20–30% of previously untreated patients (PUPs) with severe HA, and in 1–5% patients with HB [3]. Both the short coagulation factor half-life and the inhibitor formation represent a big challenge in the treatment of hemophilic patients, as they significantly increase healthcare expenses and patient morbidity. Since inhibitors occur more frequently in HA and the mechanisms responsible for their formation are different between HA and HB, this review will focus principally on immune tolerance in HA.

Many genetic and environmental factors have been linked with the risk of antibody formation against FVIII [4], highlighting the complexity of the process. The genetic factors include FVIII mutations, ethnicity, and polymorphisms of immune-genes, while the environmental factors include the origins of the administered replacement product, the age of the first injection, the dosage and the clinical state of the patient [5]. The total absence of FVIII in hemophilic patients carrying null mutations in *F8* gene confers the highest genetic risk [6] due to the lack of central tolerance toward the clotting factor.

Nevertheless, peripheral tolerance may be involved since α -FVIII antibodies [7] and specific-FVIII T cell clones [8] were found in healthy subjects. The peripheral immune response against FVIII requires first internalization and degradation by antigen presenting cells (APCs), followed by the presentation to CD4⁺ T cells in the presence of pro-inflammatory cytokines. Activated CD4⁺ T cells stimulate in turn FVIII-specific B cell clones to secrete α -FVIII antibodies [9]. The FVIII neutralizing antibodies found in patients are mainly IgG4 and IgG1 isotypes [10]. Due to their role in tolerance modulation, T regulatory cells (Tregs) have been shown to play an important role in controlling the self-reactive α -FVIII T and B cell immune response. As described below, many pre-clinical studies have targeted Tregs for inducing tolerance, especially in a setting in which inhibitors have already developed. Clinical studies indicate that on-demand treatment and surgery affect antibody development in accordance with the need of a concomitant danger signal [11] to elicit a humoral response. FVIII is unique in its immunogenicity since most soluble proteins normally fail to raise an immune response when intravenously (IV) injected without adjuvants. FVIII is heavily glycosylated and circulates bound to von Willebrand Factor (vWF), which may confer protection from the immune system by shielding possible epitopes [12] and regulating the vWF-FVIII complex clearance [13]. This immune-protective role is supported by a study reporting a reduced incidence of inhibitor formation in pdFVIII compared with rFVIII-treated patients [14]. Indeed, post-translational modifications such as glycosylation are of human origin for pdFVIII, while they are different for rFVIII produced in non-human cell lines (baby hamster kidney [BHK] or Chinese hamster ovary [CHO]). The contribution of the origin of FVIII products to the immune activation, however, remains controversial [2]. Another meta-analysis reported no

difference between pdFVIII and rFVIII but a higher risk in second- compared with third-generation recombinant products [15]. The latter ones are obtained from productive systems deprived of any animal or human proteins. Experimental and computational analyses for designing less immunogenic FVIII molecules have also been proposed for preventing inhibitor formation. While these approaches have been recently reviewed elsewhere [16], this review will describe new methods based on cell- and gene-therapy and on the manipulation of the immune cells.

2. Current gene therapy clinical trials for correcting hemophilia

Rather than a replacement therapy based on the repetitive infusion of either FVIII or FIX, gene therapy represents a very promising approach for long-term treatment of hemophilia. Indeed, the disease is due to mutation/s in a single gene and the coagulation factors infused or expressed by gene transfer are normally active in the bloodstream, with a 20–40% activity enough to achieve clinical correction since it prevents spontaneous bleedings and does not require prophylactic FVIII injections. Moreover, as the clotting factor requires only to be secreted into the blood, different cells can be targeted for the genetic correction and not necessarily the ones physiologically responsible for their secretion (liver sinusoidal endothelial cells (LSEC) for FVIII [17–19] and hepatocytes for FIX [20]).

Since 1989, more than 2500 gene therapy clinical trials have been conducted. Despite several initial setbacks, to date both positive and safe results have been documented for many genetic diseases, including Wiskott-Aldrich syndrome (WAS), β -thalassemia and adrenoleukodystrophy (ALD), and cancer immunotherapy which employs chimeric antigen receptor(CAR)-modified T cells [21,22]. Gene transfer approaches for hemophilic patients have been continuing for 20 years [23]. At present, there are five active clinical trials for HA and seven for HB [24] that employ adeno-associated virus (AAV) vectors.

AAV vectors are used for gene therapy due to their relative safety, simplicity and tissue-specific tropism [25]. One drawback is the presence of AAV-specific antibodies in 30–40% of the population, even if AAV infection has no relevant clinical effect. Those antibodies could potentially inhibit *in vivo* transduction and, therefore, decrease the gene transfer efficiency [26]. Moreover, AAV-injected patients typically develop antibodies against the vector limiting the possibility of a second injection. Most of the viral DNA does not integrate into the host cell genome, therefore, proliferating cells will lose the transgene limiting the long-term efficacy of the therapy. As described, all the approved clinical trials for hemophilia use AAV, despite the fact that the size of the F8 gene raises a big challenge for packaging inside this vector [27]. The AAV genome is ~ 4.7kb in size and, therefore, is unable to accommodate the full length FVIII cDNA (9kb). Ongoing clinical trial for HA [28] contains the B-domain deleted FVIII (4.45kb), which is easily packaged inside an AAV.

Lentiviral vectors (LVs) represent an alternative vehicle for gene therapy that has already been approved in clinical trials [22]. The major advantages for using LVs are their ability to

integrate their DNA into the host genome, the lack of viral genes in the transfer construct, and the size available for expression cassettes (up to 7 kb). LVs have been largely investigated *in vitro* and in pre-clinical models for correcting hemophilia genes and different approaches have been tested for inducing immune tolerance to FVIII or FIX [29–32].

3. Gene therapy approaches for inducing immune tolerance in hemophilia A

To date, all gene therapy clinical trials approved for hemophilia employ an *in vivo* approach, where the corrected gene combined in a viral vector, is delivered by IV injection. It is also possible to apply an *ex vivo* method, by combining gene and cell therapy. This involves the isolation of patient target cells, their correction by *in vitro* transduction, and re-implantation in the patient. Both methods allow for the development of strategies which favor tolerance to the transgene product. An alternative strategy to prevent inhibitor formation is to directly manipulate immune cells to induce tolerance.

3.1. *In vivo* liver directed gene therapy

The liver has been the main organ targeted for *in vivo* gene therapies for HA, since it is the main source for coagulation factors and for its immune-modulatory environment [33]. The use of cell- and/or tissue-specific liver promoters is helpful for directing liver transgene expression in the organ or in specific hepatic cells (e.g. hepatocytes or LSEC). Tolerance induction upon liver gene therapy has been shown to be related to Treg activity and consequently suppression of both humoral and cytotoxic-specific responses [34]. Several pre-clinical studies in adult dogs have confirmed the low incidence of inhibitor formation with FVIII correction by *in vivo* AAV-liver directed delivery, even in presence of pre-formed specific antibodies [3]. These data have been further confirmed by the absence of inhibitor development in patients currently enrolled in ongoing gene therapy clinical trials. For testing the feasibility of this therapeutic approach in pediatric patients, newborn hemophilic mice were treated with AAV carrying FVIII on day two after birth [35]. The results were promising since all treated mice displayed FVIII correction and no immune response for up to 22 months.

Safe and durable liver gene transfer have been shown in pre-clinical studies employing LVs

[29–32]. In contrast to AAV, most people have not been exposed to HIV and thus the use of LVs should reduce the probability of eliciting an immune response against the viral particles. Liver specific promoters have been extensively described and exploited for treating hemophilia, while only recently one study demonstrated therapeutic benefits in HA mice by targeting hepatic endothelial cells (ECs) using an endothelial-specific promoter (vascular endothelial cadherin, [VEC]) without inhibitor appearance [32]. Tregs were shown to play a major role in the tolerization process as their *in vivo* depletion brought about a decrease in FVIII activity and an increase of inhibitors. Based on this study, it is evident that an important interaction takes place in the liver between LSEC and Tregs for promoting tolerance. Overall, a deeper

understanding of the molecular and cellular mechanisms conferring to the tolerogenic ability of LSEC in relation with Tregs and effector T cells should be instrumental in the design of new strategies for the achievement and maintenance of immune tolerance.

A new approach for preventing off-target expression of the transgene in cells able to activate the immune response has focused on the fine-tuning regulation in cell expression offered by the insertion of miRNA target sequences (miRTs) to the transgene cassette. By engineering the vector to avoid endogenous transgene expression in professional APC, such as Kupffer cells in the liver, researchers were able to obtain both a cell-specific expression and an enhanced immune tolerance [29,32,36].

Prevention of immune reactions can be obtained directly *in vivo* by co-delivery of FVIII and the indoleamine 2,3-dioxygenase (IDO) gene via a transposon iv injection into HA mice [37]. IDO is an enzyme active in tryptophan metabolism and it acts very strongly in modulating the immune responses toward tolerance. If the co-delivery of IDO gene with the therapeutic one is difficult to establish, it may be more feasible to upregulate IDO expression by administering TLR9 agonists [38] after vector administration.

3.2. Ex vivo gene correction for hemophilia

Healthy bone marrow (BM) cells were able to partially correct a bleeding phenotype in HA mice [39] indicating that BM-derived cells were able to secrete therapeutic levels of FVIII. However, allogeneic BM transplantation cannot be introduced for treating hemophilic patients due to the related toxicity and possible complications. Autologous hematopoietic stem cells (HSCs) could be an alternative target for introducing the corrected gene, as proven in some hematological diseases, such as β -thalassemia [40] and Fanconi anemia [41]. HSC transduction requires an external *in vitro* manipulation while transplantation regimens conditioned by drug administration could bias the immune tolerance evaluation. More promising is restricting FVIII [42] production in platelets by *ex vivo* LV transduction of HSCs with a cassette carrying a platelet-specific promoter. This averts transgene expression in professional APC and, therefore, promotes immune tolerance, even in the presence of pre-existing antibodies. To avoid the condition regimens, recently a direct intraosseous LV delivery was performed in mice [43]. Efficiency of BM cell transduction and FVIII production was good, however, the presence of the ubiquitous EF1 α promoter led to high inhibitor titer. On this basis, they switched to a megakaryocytic promoter, Gp1ba, which maintained a long-term bleeding phenotype correction with no inhibitor development. This confirmed the importance of targeting the right cell type for obtaining the immune tolerance.

Cells from BM have been widely investigated for gene correction due to their relatively easy accessibility and the extensive knowledge acquired for BM transplantation. Despite this, numerous other cell sources have been used for combining gene and cell therapy for a hemophilia cure [44]. FVIII-corrected fibroblasts were the first to be approved for clinical trial, however, FVIII secretion was limited overtime [45].

ECs represent an appealing population at least for correcting FVIII deficiency, as they are the main producer of FVIII and they strictly interact with immune cells. In particular, peripheral blood outgrowth endothelial cells (BOECs) have been identified as a good candidate for gene correction, as their *in vivo* re-implantation sustains FVIII production in the absence of neutralizing antibody development [46,47].

Corrected cells can be injected at the sites where the clotting factor is needed more. In fact, autologous FVIII-corrected mesenchymal stem cells (MSCs) administered to the joints of hemophilic mice, improved the hemarthrosis and prevented new bleeding [48], with no appearance of inhibitors. Indeed MSCs have been recognized as cells with a high tolerogenic potential [49], and they express and secrete FVIII [39,50].

4. Tolerance induction in hemophilia by manipulation of immune cells

Another approach for inducing tolerance to specific antigens is to act directly on the cells involved in the immunological processes. Many studies have been focusing on Tregs, due to their role in preventing auto-immune reactions. The main challenge is to obtain and expand specific Tregs, which should be able to block *in vivo* T effector activation and B cell antibody secretion. Indeed, one group showed high suppression activity both *in vitro* and *in vivo* of Tregs retrovirally transduced with a FVIII-specific TCR isolated from an HA patient [51]. This strategy is limited due to the need of matching the HLA system to obtain the correct interaction with the specific TCR, while the new technique for generating CAR eliminates this problem. Tregs with α -FVIII-specific CAR molecules were generated by LV transduction and showed a successful tolerogenic behavior [52]. *In vivo* expansion of Tregs could be obtained by injection of different immune-modulatory molecules before or at the same time of FVIII injection or correction. Interleukin2 (IL2) complexed with IL2 monoclonal antibody [53], rapamycin [54] or dexamethasone [55] administration, promoted FVIII-specific tolerance by increasing Treg numbers.

As B cells are the source of inhibitors and efficient APCs, several groups decided to engineer them for obtaining immune tolerance. B cells were initially transduced employing retroviral vectors with the aim being to introduce IgG fusion proteins with FVIII domains (e.g. A2 and C2) [56] or FIX [57]. In both cases, the modified B cells were able to prevent or negate neutralizing antibodies. Specific B cell targeting was improved by designing an envelope expressing a single-chain variable fragment recognizing the CD20 molecules [58], or by introducing a baboon retroviral envelope glycoprotein (BaEV) instead of the common vesicular-stomatitis-virus G (VSV-G) [59], in the design of a hybrid LV. A promising strategy combining specific α -CD20 depletion of B cells with rapamycin for reverting inhibitor formation in a mouse model of HA [60], highlighted the importance of targeting B and T cells together for achieving full tolerance.

Dendritic cells (DCs) are professional APCs often associated with immune response activation, but in general they exert an

immunomodulatory activity. Mouse BM DCs cultured in conditions promoting their tolerogenic capability and transduced with a foamy virus vector harboring both FVIII and IL10, suppressed T effector cells and decreased inhibitor levels [61].

5. Expert commentary and five-year view

Gene transfer in the liver has achieved the stable and functional activity of a coagulation factor in the absence or eradication of pre-existing inhibitors. Both pre-clinical and clinical studies demonstrate the stability and safety of liver gene therapeutic approaches, however, very little is known about the long-term effects in humans, despite pre-clinical models in dogs demonstrating FIX secretion throughout their lives [3]. In the near future, it is important to extend the clinical success achieved in adult hemophilic patients to pediatric patients, who represent the ultimate target population that can most benefit from gene therapy. To date, AAV has not been used in hemophilic children or infants due to the limited knowledge available on the maintenance of the transgene in tissues characterized by high rates of proliferation. In contrast to AAV, LV integrate into the target cell chromatin and are maintained as cells divide, however, liver disease or injury may occur in gene therapy treated patients, leading to loss of transgene production and/or specific tolerance. Studies with a long-term follow-up are needed to elucidate whether vector transduction can last for all the patient lives without increasing the tumor incidence. Therefore, it would be important to perform more pre-clinical investigations to assess the proliferation and turnover of different hepatic cell populations (e.g. hepatocytes, LSEC and Kupffer cells) during postnatal liver growth, in homeostatic renewal and in response to different types of liver damage. These results will guide future research in improving AAV and LV design to mediate gene therapies targeting the liver and sustaining long-term tolerance to the corrected gene.

Both *in vivo* and *ex vivo* LV-based transduction therapies require an extremely long follow-up to confirm the absence of insertional mutagenesis. A recent review listed in detail the regulatory elements established by the Food and Drug Administration (FDA) for the ongoing clinical trials [62]. Currently, the use of LVs *in vivo* in clinical settings is still limited due to the difficulty in scaling up the good manufacturing practices (GMP) production required for obtaining the high vector titers for human treatment [63]. However, a new stable inducible producer cell line was recently developed with cells cultured in suspension in a bioreactor which allowed production of LV titer suitable for human gene therapy applications [64]. More interesting, a new packaging lentiviral system has been built to overcome the risk of triggering immune responses after vector delivery. The beta-2 microglobulin gene was disrupted in the producer cell line obtaining MHC-free LVs [65]. These LVs are able to efficiently transduce target cells and are more resistant to complement-mediated inactivation compared with LV produced by the classical transient transfection method.

Gene transfer directed to cells recovered and then re-infused into the patients has the advantage of avoiding an immune reaction against the injected viral particles and can

strictly control targeted cells. Induced pluripotent stem cells (iPSCs) have largely emerged as a source for cell therapy approaches. They can be obtained from patients, corrected *in vitro* by gene transfer, differentiated, expanded, and administered freely or via specific matrix/devices which facilitate cell engraftment and vascularization, and prevent an immune response, as it has demonstrated for type 1 diabetes [66]. Several protocols have been established for differentiating iPSCs into the desired mature cells and for the success of the hemophilia therapy it is important to choose cells with the highest capability to physiologically secrete the lacking clotting factor and to prevent or even abolish immune reactions. For FVIII, ECs represent an attractive target, as highlighted by pre-clinical studies [32,47]. Although very promising, clinical application of this strategy would require the development of an efficient EC differentiation protocol from iPSCs, as some limitations have been reported in microvascular formation *in vitro* for iPSC-derived ECs [67].

Recently, a different approach for immune system modulation has been introduced aiming to achieve oral tolerance through development of transgenic plants [68]. Bioencapsulated delivery of FVIII domains successfully suppressed and reverted inhibitor formation in hemophilic mice [69]. Induction of oral tolerance to FVIII is appealing since it does not require injection or genetic manipulation, however, it may require repeated administrations. Oral tolerance induction is controlled first by the gut environment and second by the liver. Microbiota and food intake could strongly influence the immune reactivity against self and non-self antigens [70]. Thus, it might be relevant to perform new studies on the microbiota involvement that can influence immune tolerance toward FVIII in patients treated with gene or replacement therapy.

In the years to come, several novel strategies for genome editing may be translated to a treatment for hemophilia. Manipulations of cells and organisms using programmable nucleases (e.g. TALEN, CRISPR/Cas9) are in constant expansion and have recently reached the clinic [71]. A study on human iPSCs have shown the feasibility of introducing specific inversions in *F8* gene [72] while Guan *et al.* [73] were able to correct the *F9* gene *in vivo* by CRISPR/Cas9 delivery. They compared naked DNA versus AAV injection of Cas9 elements in a mouse model of HB, reporting a reduced correcting efficiency for the former which was compensated for by a better overall therapeutic effect due to the absence of liver toxicity. More recently, correction of the *F9* gene was shown in neonate mice by the administration of CRISPR/Cas9 system, highlighting a possible application in young patients who are currently excluded from gene modification therapies [74].

With the number of groups studying novel strategies and the rapid improvements in terms of both safety and efficacy reported in recent years for gene therapy manipulations, in the near future more patients will be treated via a gene manipulation approach. While a full safety evaluation will not be established in the short-term, the possibility to provide with one infusion the stable and safe secretion of the clotting factors is an appealing goal which will provide a huge improvement in the morbidity and a patient's quality of life.

Key issues

- Liver gene therapy is a promising strategy for overcoming two main limits of the actual hemophilia replacement treatment: the short coagulation factor half-life and the inhibitor formation.
- Gene therapy vectors should carry both transcriptional (e.g. cell specific promoter) and post-transcriptional regulatory elements (e.g. miRTs), useful for enhancing specific coagulation factor production and preventing immune activation.
- Gene and cell therapies may be combined for obtaining the best result in establishing the long-term expression of the corrected gene and the induction of FVIII tolerance.
- Both humoral and cytotoxic arms of the immune system need to be regulated for successfully preventing and/or eradicating specific-FVIII immune responses.
- Genome editing by use of programmable nucleases (e.g. TALEN, CRISPR/Cas9) represents a potential new approach with high potential for correcting gene mutations causing diseases.

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Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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