

Factor VIII: Perspectives on Immunogenicity and Tolerogenic Strategies for Hemophilia A Patients

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A major complication in treating hemophilia A is the development of neutralizing antibodies (inhibitors) against therapeutic administered factor VIII (FVIII), which occurs in approximately 20-30% of patients with severe disease. These inhibitors render FVIII replacement therapy ineffective and increase the morbidity and mortality risk. The currently accepted method to eradicate inhibitors is immune tolerance induction (ITI), and frequent intensive administration of FVIII until inhibitor titers drop. Current ITI protocols are extremely costly and not effective in all patients. During the last decade, many types of research have been accomplished to clarify the mechanisms that mediate immune tolerance induction. Novel experimental therapies including monoclonal antibodies, viral vector-mediated gene therapy, regulatory T cell induction using immunosuppressive drugs, and nanoparticle-based immune modulation show promising results in hemophilia A clinical trials. This review focuses on treatment options towards the anti-FVIII immune responses and current novel therapies in clinical trials.

Key words: Factor VIII, hemophilia A, inhibitors, immune tolerance

Hemophilia A is an X-linked disorder which is associated with recurrent bleeding in affected patients. The prevalence of hemophilia A is 1:5000 of live male birth without any genetic predisposition (1). This life-threatening disorder is caused by a functional defect in plasma coagulation factor VIII (FVIII) in human. For many years, administration of this protein as a plasma or recombinant product has been used as a treatment for hemophilia (2, 3).

Alternative transfused FVIII has been a practical treatment; however, developing antibodies against this protein, which play a role as inhibitors,

has restricted its application. Inhibitor development predominantly occurs in approximately 30% of all patients with severe hemophilia A and 5% of all patients with mild hemophilia A (4). A series of both genetic and non-genetic factors are involved in FVIII inhibitor development. Mutations at specific regions of *F8* gene (hot spots, heavy chain coding regions) are associated with delayed or absent pro-coagulant activity. Patients with large deletions and non-sense mutations should lack tolerance to all of the epitopes in FVIII, and patients carrying small deletions/insertions and missense mutations are associated with mild or moderately severe

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hemophilia A. Findings from a series of studies showed a significant association between the HLA class II molecules and inhibitors development. However, the strength of this association depends on the genetic background of different populations (5). For instance, Hosseini et al. found a strong association between HLA-DRB1*01:01 allele and a lower risk of developing inhibitor in Iran (6). Actually, due to the development of these inhibitor antibodies in responder patients, the FVIII replacement therapy is not an efficient and cost-effective treatment (7). Consequently, ineffective treatment may lead to worsening of the situation through bleeding and consequent high morbidity rate increases disability and decreases quality of life (8).

Given the significant burden of inhibitors on both patient's health and health-care costs, many efforts have been made to prevent anti-FVIII antibodies. Immune tolerance induction (ITI) is the most successful approach which consists of intensive high dose FVIII treatment (9-11).

Although ITI has been effective in 60-80% of the patients, and is known as a preventive treatment for complications following the inhibitor development, it is extremely expensive and it often takes several years in to achieve an effective tolerance (12, 13). Accordingly, there is still a compelling need for cost-effective treatment with the rapid and productive clinical results in inducing tolerance against FVIII. (14).

In this review, we discuss the characteristics of factor FVIII structure, and the pathophysiology of inhibitor formation. In addition, several novel approaches to modulate the immune response and induce tolerance are described.

Factor VIII structure and function

FVIII is a non-covalent heterodimer protein, which is encoded by the *F8* gene. The immature FVIII protein consists of 2351 amino acids (aa) comprising a mature protein of 2332 aa and a signal protein of 19 aa. As Figure 1 shows, this multi-

domain protein comprises a heavy chain (A1-A2-B domains) and a light chain (A3-C1-C2 domains) (15).

FVIII endures several post-translational modifications, including a high glycosylation process before becoming ready to circulate as a prepared glycoprotein. The heterodimer FVIII a high affinity ($k_d \sim 0.3$ nM) for von Willebrand factor (VWF), which is a chaperone molecule acting through the blood circulation (16).

The VWF-FVIII complex is formed through non-covalent interactions between A3, C1 and C2 domains of FVIII and the D'D3 domains of VWF. Actually, VWF is essential for the maintenance of the heterodimer structural stability of FVIII, and increases the stability of the interaction between the FVIII heavy and light chains and also increases the FVIII half-life. In addition, the interaction of VWF with FVIII is beneficial for primary hemostasis, so it increases FVIII involvement in vascular injury.

Activated FVIII (FVIIIa) is released from VWF following proteolytic cleavage and release of the B-domain. (17, 18). The formation of FVIIIa is crucial for the coagulation cascade due to the important role of FVIIIa as a cofactor for factor IXa protein. In fact, intrinsic Xase complex, which is essential for thrombin formation is normally generated as a result of the factor IXa function with the aid of its cofactor, FVIIIa. Endogenous FVIII has a half-life of 12–16 h, after which FVIII is eliminated by the liver and probably also by the spleen. Of note, the life cycle of therapeutically administered FVIII is similar to endogenous FVIII. However, the interaction between endogenous VWF and FVIII normally takes place in the circulation instead of the liver (19, 20).

Molecular and clinical aspects of inhibitor formation

Definition of an inhibitor

The immune system in responder patients produces polyclonal high-affinity IgG antibodies against FVIII which are known as FVIII inhibitors.

This is a T cell-dependent immune response in which associated innate and adaptive immune cells, including antigen-presenting cells (APCs), B and T-helper lymphocytes are involved (21).

Although FVIII protein contains many functional epitopes which can potentially interact with specific inhibitors, antibodies targeting the A2 and/or C2 domains of FVIII are the most frequent (22).

Recognition of the above-mentioned epitopes by antibody gives rise to the blockade in functional domains of the FVIII protein, which are normally used to interact with FIX, phospholipid and VWF factor interaction sites (23). Different targets of FVIII epitope can be recognized by the inhibitors at the same time, and these epitope targets can change

over time (24). In addition, some anti-FVIII antibodies have the ability to play a role as a hydrolytic enzyme and inactivate the FVIII (25).

According to the kinetics and severity of inhibitory functions, anti-FVIII antibodies have been classified to two groups, including type I and II. Type I inhibitors are able to thoroughly inactivate FVIII based on their second-order kinetics (dose-dependent linear inhibition). They are more common in patients with severe hemophilia. Conversely, type II inhibitors, due to their complex kinetics, inactivate FVIII in an incomplete way, and are commonly developed in patients with mild hemophilia or who develop acquired FVIII inhibitor (26).

Immune response to FVIII

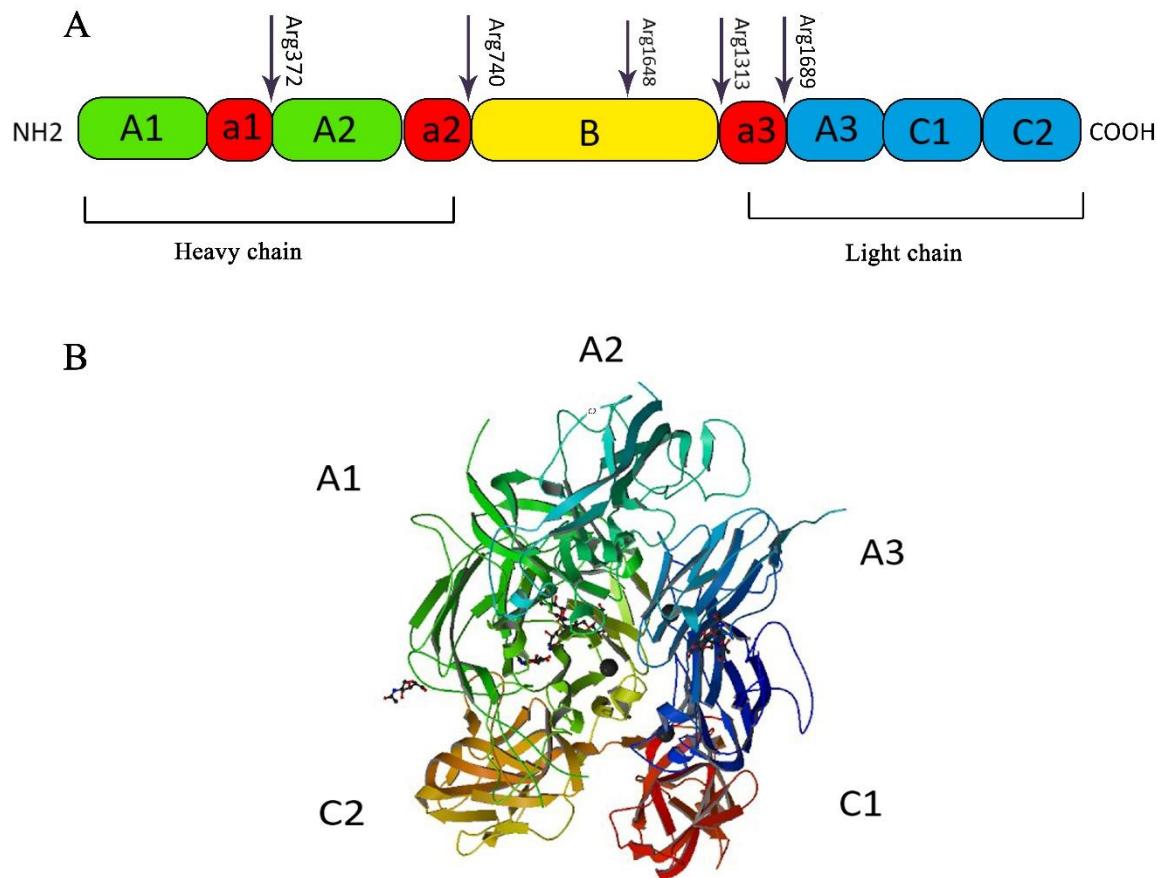


Fig. 1. Factor VIII structure and domain organization. A: linear arrangement as well as activation cleavage sites of the FVIII domains A1, A2, B, A3, C1, C2 are shown; B: three dimensional crystal structure of B-domain depleted human factor VIII was created using RCSB PDB viewer.

Recently, many review studies have discussed several aspects of the immune response against FVIII which can be the result of considerable advances in this area (27, 28). FVIII proteins, at the first encounter with the immune system, are predominantly recognized and captured by professional antigen presenting cells (APCs) including dendritic cells (DCs), macrophages, and B cells. Following internalization of FVIII into APCs, it undergoes proteolytic cleavage in the endosomal compartments. Afterwards, small peptides derived from a large FVIII protein are presented on the surface of APC through major histocompatibility complex (MHC) class II. The MHCII-peptide complex is then recognized by naïve T cells present in the lymph nodes (29). To effectively respond to the FVIII peptides, naïve T cells should be differentiated into specialized effector CD4⁺ T cells. In addition to the MHCII-peptide complex, there are several co-stimulatory molecules engaged in the effector T cell development. CD40 and CD80/86 (B7-1/B7-2) molecules on the surface of APCs come into a strong interaction with CD40 ligand (CD40L) and CD28 on the surface of naïve helper T cells (CD4⁺), resulting in the secretion of cytokines by both DCs and T cells (30). Interestingly, when human DCs are cultured with FVIII, DCs do not mature (31). The cause of this difference in *in vitro* and *in vivo* immune system recognition of FVIII is unclear, but probably the environmental differences, including danger signals resulting from trauma, surgery, severe/recurrent bleeds, infection or vaccination may influence the risk of immune reaction towards administered FVIII in hemophilia A patients (32, 33). The physical characteristics of the FVIII antigen, such as post-translational modifications or physical aggregation in high administered doses, may have a significant effect on its immunogenicity (34). Differences in the intrinsic or extrinsic property of the antigen, could affect the immune response against administered FVIII. This may be

due to glycosylation patterns depending on their cell expression system and covalent modifications to increase the circulating half-life of the protein (Fc fusion, PEGylation etc.) (35, 36).

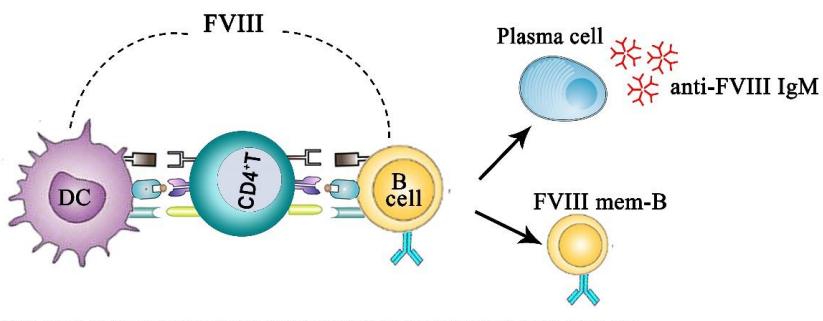
As both humoral and cellular arms of the immune system are involved in an effective immune response, T cell activation gives rise to B cell differentiation, proliferation, and class switching. As a consequence, B cell activation results in producing large amounts of FVIII antibodies that block the FVIII function. Moreover, memory B cells and specialized plasma cells are also ready to intensify the battle (22). During the secondary immune response, T cell and B cell activities are intensified, and higher amounts of specific antibodies which have already undergone the affinity maturation process are produced from plasma cells (Figure 2). Most of identified antibodies against FVIII epitopes in hemophilia patients are immunoglobulin G which IgG1 and IgG4 are the most prevalent subclasses. IgG4 production needs a further class switching process that proves the involvement of T cells in the formation of an effective immune response against FVIII (37).

As it has been shown in murine hemophilia models, the presence of co-stimulatory interactions and signals is very important to form a protective immune response. Actually, the lack of a functional co-stimulatory signal gives rise to anergy or apoptosis of the T cells. A defective immunologic synapse in the absence of co-stimulatory interactions produces tolerogenic T cells that do not react with the antigens presented by the APCs (38, 39).

Immune tolerance induction

ITI is the most prevalent method exploited to prevent complications related to FVIII infusion (11, 40). As mentioned before, frequent exposure of the immune cells to FVIII antigens in a non-inflammatory environment can lead the immune system response toward anergy and tolerance. How-

A. Primary Response



B. Secondary Response

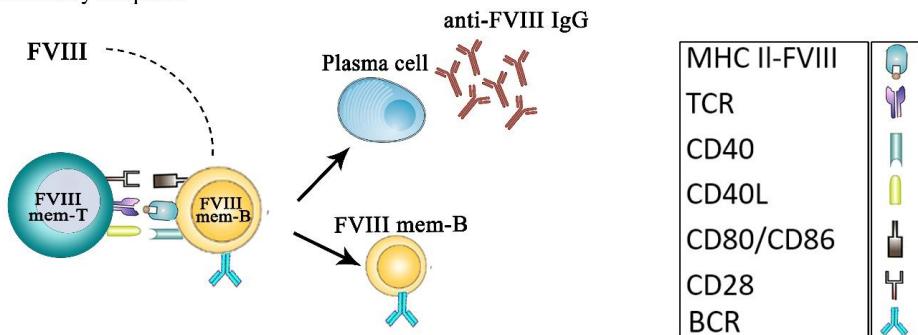


Fig. 2. Primary and secondary immunization in response to FVIII. A: primary response; upon the first exposure to FVIII, this protein is internalized by DCs or other APCs; then the protein is presented to naive CD4⁺ T cells which results in its activation in the presence of co-stimulatory signals. The activated T cell activates naive B cells that expand and differentiate into FVIII plasma cells, secreting anti-FVIII IgM antibodies, or FVIII memory B cells. B: Secondary response; in this type of immunization, FVIII memory B cells act as APCs and activate memory T cells, and then memory B cells will differentiate into FVIII plasma cells that secrete anti-FVIII IgG antibodies. DC: dendritic cell; APC: antigen presenting cell; MHC: major histocompatibility complex; TCR: T-cell receptor; CD40L: CD40 ligand.

ever, the mechanism of this tolerance induction is not completely understood. Through the ITI method, regular administration of FVIII makes the immune cells to ignore FVIII antigens and develop a tolerogenic response against it (41). To reach the most practical ITI treatment, many different regimens such as encompassing protocols with variations in FVIII dose and using additional immunosuppressive agents have been tested. From all of them, Bonn, Van Creveld, and Malmö ITI protocols are mostly accepted and used, and underwent various modifications. It has been shown that the immune modulation effectively increases the result of the treatment (42).

Elucidating the mechanisms leading to functional immune tolerance and specific reduction or elimination of inhibitor responses are the common destinations of all investigations on ITI therapy to develop a promising treatment (43).

However, due to the high cost, moderate success rate, long duration and inconvenience of daily infusions, ITI therapy is not a method to be used extensively. In order to bring better results, all of these complications highlight the need for further modifications (44).

There are three mechanisms which all contribute in inducing immune tolerance:

1. Development of monoclonal antibodies (mAbs) as the specific immuno-suppressant
2. Anergy of effector T-cells due to the induction of Tregs using chronic exposition of FVIII
3. Inhibition of memory B cells differentiation into plasma cells using high FVIII concentrations

Development of monoclonal antibodies (mAbs)

A recent investigation has shown that mAbs can be used as specific immunosuppressive agents that appear to be both more effective and more selective in facilitating immune tolerance induction,

and they are generally well tolerated by recipients. Actually, these immunosuppressive agents pointing at specific targets have less toxicity in comparison with the immunosuppressive agents that target many aspects of the immune system. Hence, they have considerably lower side effects, and can be better tolerated by the recipients. There have been many studies on FVIII knockout mice using mAbs affecting a myriad of immunological pathways. The outcome has shown successful tolerance induction, especially when co-administered with antigen (45-47). Several investigations have displayed that blockade of co-stimulatory molecules' responses, including CD40/CD40L and B7/CD28 can effectively diminish the inhibitor development in hemophilia A mice models. It has been shown that mAbs against CD40L prevented anti-FVIII antibodies induction, with suppression of FVIII specific T-cell responses in FVIII-primed hemophilia A mice (48, 49). In addition, blockade of B7/CD28 using specific antibodies against CTLA4 (CTLA4-Ig) effectively obstruct the inhibitor development in a mouse model of hemophilia A (50).

Another study on FVIII-plasmid treated hemophilia A mice revealed that dual blockade of CD40/CD40L and B7/CD28 pathways using combined anti-CD40L and CTLA4-Ig obstruct both associated co-stimulatory signals in a synergic manner, and effective long-term tolerance was obtained against FVIII (51, 52).

T cell anergy and Treg induction

In addition to co-stimulatory molecules, mAbs targeting pan T cell markers, especially CD3 have been shown to effectively increase T cell apoptosis. Apparently, T cell depletion decreases the cellular immune response against the antigen of interest (53).

As a study on hemophilia A mice revealed, that anti-CD3 treatment, concomitant with FVIII-plasmid injection prevent inhibitory antibodies and persistent FVIII expression levels was achieved.

The mechanism involved in this process is increasing immunomodulatory cytokine TGF- β that is a key factor in decreasing cellular immune recognition of FVIII antigen. Consequently, a long-term tolerance was formed against FVIII, and the inhibitor development was controlled. Besides, administration of anti-CD3 alone diminished pre-existing antibodies against FVIII (54).

Regulatory CD4 $^{+}$ CD25 $^{+}$ T cells play a prominent role in homeostasis of T cells. Actually, they balance the cellular immune response by suppressing effector T cells after a protective immune response against an antigen. T cell hemostasis through Treg activity is very important to protect the body against complications followed by an uncontrolled immune response including autoimmunity or alloimmune responses. This characteristic of regulatory T cells has been exploited to induce a long-term immunomodulatory response against FVIII (55). In fact, a higher number or percentage of CD4 $^{+}$ FOXP3 $^{+}$ Tregs in both protein replacement and/or gene therapy settings are used to control inhibitor formation even in patients with a measurable pre-existing inhibitor titer (56, 57). These regulatory cells have been shown to effectively restrict both cellular and humoral immunity through suppressing cytotoxic CD8 $^{+}$ T cell activity and antibody production by B cells in mice and non-human primates, respectively (58, 59). In addition, Fas/FasL interaction and T cell depletion were also studied in Fas-deficient mice to highlight the importance of Treg activity in the formation of a robust immune tolerance (60).

Regulatory T cells use different mechanisms to prevent differentiation of T cells into effector cells and promote their conversion into Tregs. They can either directly contact the target cells or indirectly reduce their activity by producing immunomodulatory elements comprising IL-10 and/or TGF- β cytokines (61). It has been shown that using antigen together with the mTOR inhibitor Rapamycin can effectively reduce the effector T

cells and increase the regulatory lymphocytes instead (Figure 3-A) (62).

IL-2/IL-2 mAb complexes are a good alternative method to enrich Treg *in vivo* and rapid formation of CD4⁺CD25⁺FOXP3⁺ Tregs. These Tregs are highly effective as a pre-treatment for preventing autoimmunity or transplant rejection due to their strong suppressive activity (63). IL-2/IL-2 mAb complexes were also used in a hemophilia A mice models and caused a considerable reduction in FVIII inhibitor titer to either FVIII replacement therapy or plasmid-mediated gene therapy. It has been proven that these Tregs induce a lasting

tolerance against FVIII protein, which was generally due to their ability to convert from FVIII-specific CD4⁺ CD25⁻ conventional T cells into Tregs, in the presence of TGF- β 1 (64, 65).

Therefore, producing a higher number of regulatory T cells with specificity to FVIII through redirecting antigen-specificity through TCR or chimeric antigen receptor (CAR) gene transfer to Treg is extensively attractive (Figure 3-B). The investigations showed that the expression of a single human TCR, using *ex vivo* retroviral gene transfer, can negatively affect CD4⁺ T cell and B cell activity, and also suppress hemophilia A

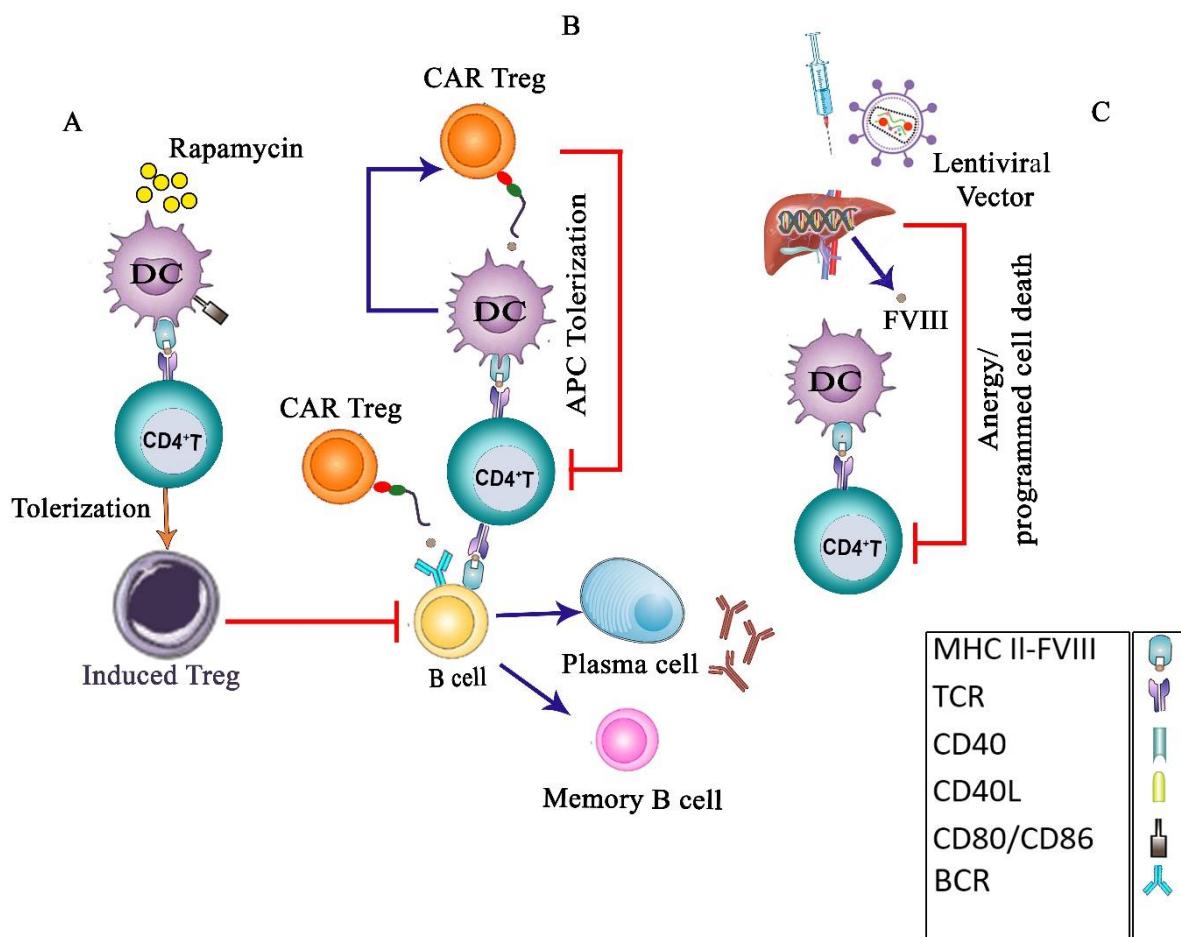


Fig. 3. Significant approaches for inducing tolerance and eliminating inhibitor formation in hemophilia A. A: selective deletion of CD4⁺ T-helper cells and Tregs induction occur upon administration of Rapamycin as an immuno-suppressant; B: CAR Tregs are engineered using fusion of TCR signaling molecules and scFV domains. CAR Tregs become activated through recognition of FVIII presented by DCs. These pathways result in CD4⁺ T cells suppression and APC toleration; C: hepatic lentiviral gene transfer is known as a way for tolerance induction which results in programmed cell death of CD4⁺ T-helper cells and the Treg induction. CAR: chimeric antigen receptor; scFV: single-chain variable fragment; Treg: regulatory T cell.

mice expressing human HLA (66). Actually, the induced regulatory T cells predominantly react against only a single epitope. However, this recognition can lead to a general suppression against the FVIII molecule entirely. Due to highly extensive HLA differences, the production of Treg against every single immuno-dominant epitope in FVIII protein requires many TCR clones. Hence, the CAR approach came to streamline general Treg production, which can be used for all patients as it is not MHC restricted, and consequently antigen recognition and cell signaling by CAR Tregs happens in the absence of APCs (67, 68). CAR T cells are predominantly produced through introducing antigen recognition variable region (single-chain variable fragment) antibody domains fused to primary and co-stimulatory signaling molecules and specialized to recognize surface antigens (69, 70). However, studies have demonstrated that experimentally produced FVIII-specific human CAR Tregs can provoke a suppressive response against soluble FVIII and decrease the inhibitor development both *in vitro* and *in vivo* in hemophilia A mice (71). However, the exact mechanism is not completely understood. In fact, using immunotherapy methods with the aid of genetically engineered CAR T cells in leukemia patients broaden the scientists' perspectives about possible options of exploiting FVIII-specific CAR Tregs to induce immuno-suppression in hemophilia A patients (72, 73). The presence of APCs is essential for *in vitro* suppression, highlighting the importance of cell surface interactions. A controversial question regarding this issue is whether engineered CAR Treg can suppress B cells directly or not. It is important to consider *in vivo* stability, durability of suppression and safety prior to translation of the approach.

B cell depletion therapy

In addition to T cell depletion, B cells have also been targeted due to their prominent role in producing inhibitor antibodies (74). Anti-CD20

(Rituximab) is a monoclonal IgG1 which is extensively used as immunomodulatory agent. This antibody can react with CD20 markers on the surface of follicular and marginal zone B cells, and deplete them via apoptosis. Hence, using a single dose of this immunomodulatory antibody followed by daily high-dose FVIII intravenous injections in FVIII primed-mice showed a significant reduction in the inhibitors. On the other hand, using Rituximab could affect T cells, so that increased levels of regulatory T cells were observed in the spleen after pretreatment with anti-CD20. It is worthy to note that decreased levels of B cell immune response against FVIII lasted for three months after Rituximab administration (75).

A dual therapy through which both T and B cells are targeted have been shown to bring a promising treatment to control inhibitor development in hemophilia A patients. Accordingly, Biswas et al. demonstrated that a combination of murine anti-CD20 IgG2a antibody and Rapamycin effectively decrease both humoral and cellular immune responses against FVIII, and resulted in decreased FVIII inhibitor formation in mice (76). The major side effect of both T and B cells depletion is the risk of severe infections, which needs to be taken into consideration when evaluating the risks and benefits of this therapy. Therefore, in the meantime, some degree of humoral immunity can be retained in patients by the administration of intravenous immunoglobulin (IVIG) (76).

New mechanistic insights in inducing immunologic tolerance

Hepatic gene therapy

Blocking elements for co-stimulatory molecules and inducing transient immunosuppression are not the only approaches used to solve the inhibitor problem. Administration of viral expression vectors which convey hydrodynamic naked DNA can also be used as a good strategy to form a tolerogenic environment in

order to hamper the inhibitor formation. This approach often causes rapid, but often evanescent production of functional clotting factors. However, vectors and transgenes are still non-self to the immune system, and provoke a protective immune response, especially in the type of innate immunity (55, 61, 77).

In order to treat hemophilia B, *in vivo* gene transfer to the liver was successfully performed using adeno-associated virus (AAV) vector, which is a genomic DNA covered by a protein capsid (78). As the result was successful, several liver-directed AAV vectors have been performed to find a practical treatment for hemophilia A (79). AAV vectors can be found as single-stranded DNA or self-complementary DNA genome, which are usually obtained from non-pathogenic parvoviruses (80). As these vectors do not contain viral coding sequences, they can efficiently convey genomic information in the form of DNA *in vivo*. It is important to know that the vector capacity is restricted (~5 kb) so; unessential parts of the interested gene should be omitted (81). As the B domain of FVIII does not affect the proper activity of FVIII, B domain-deleted FVIII (BDD-FVIII) is usually packaged in the vector and also used in recombinant FVIII products (82, 83).

To minimize the detrimental adaptive immune reactions, it is an effective way to use tissue-specific promoters. As these promoters usually decrease the presentation of transgenic elements in the target tissue and by APCs, they can strikingly diminish the unwanted activation of the immune system along with keeping their effective gene expression. Accordingly, there are superb options to be used in vectors for gene therapy (84-86). Liver sinusoidal endothelial cells (LSECs) are predominantly responsible of FVIII secretion into circulation, and it makes the liver to be considered as a key target for gene therapy in patients suffering from hemophilia A (87, 88). Due to the ideal characteristics of liver for the induction of immune

tolerance, including lower innate immune response, inflammatory environment, and professional APCs formation in the liver, hepatic gene transfer is an appropriate destination for different types of AAV vectors with the lowest danger of protective immune reactions (Figure3-C) (89-91). The large capacity of lentiviral vectors (LV) in gene transfer, and high potential of liver-directed gene transfer in the formation of an effective immune tolerance has proven this approach as a practical option to treat hemophilia A patients through gene therapy. The innate immune system can effectively recognize LV through toll like receptor 7 and 9, and innate pro-inflammatory cytokines, especially type I interferon, are produced following this recognition. In addition, a myriad of APCs capture and present LV antigens on their surfaces, which recruits adaptive immune responses through T cell activation and antibody production against the transgene product (92, 93).

It is noteworthy that specific microRNA (miRNA) sequences should be established in gene transfer expression cassettes due to the different possible profiles of miRNAs expression in various tissues. Actually, specific miRNA sequences (miR-142-3p) along with tissue-specific promoters considerably decreases the number of APCs with transgene expression on their surface (94, 95). Despite the fact that transcriptional and post-translational engineering of the LV can result in lower hepatocytes expression of transgenic products, exploiting tissue-specific promoters with specific miRNAs has demonstrated an effective hemophilia A and B phenotype development, and increased tolerance induction during treatment (96, 97).

Recently, Merlin et al. has tried to restrict the expression of transgenic FVIII products to certain cell types including LSECs and myeloid cells. To achieve transgene expression only in certain cell types, cell-specific promoters such as endothelial-specific promoter cadherin 5 type II, also known as

vascular endothelial cadherin (VEC) were widely used as the cell-specific promoters in a lentiviral vector (LV)-mediated gene therapy through which the immune response was restrained in a mouse model. In addition, miRT-142.3p and miRT-122 were also used as selective miRNA target sequences (miRTs) to improve specificity and achieve a silent expression of FVIII by interested cells comprising hematopoietic cells, endothelial cells, and hepatocytes (98). Moreover, the results of their studies on LV construct containing the myeloid-specific CD11b promoter, which recognizes miR-126, a miRNA that is predominantly observed in endothelial cells and plasmacytoid dendritic cells (pDCs), showed that this specific promoter restricts the expression of transgenes to the macrophages and conventional dendritic cells (cDCs) in the liver and spleen. Mice that received the vector intravenously showed only 5–6% of FVIII normal activity. This amount of activity lasted up to 1 year after vector delivery. Although the FVIII normal activity was not considerably high in injected mice, lack of antibody formation even following recombinant human FVIII use was promising (99). Therefore, expression at the physiologic site of synthesis (liver) can enhance efficacy and safety, and may help overcome the anti-FVIII immune response problem, resulting in long-term correction of hemophilia A.

Oral tolerance induction

Most of the food antigens usually do not provoke the immune reaction, and induce immune tolerance (100, 101). Hence, target antigens can be administered orally to promote tolerogenic immune response and be safe from immune recognition as dangerous agents. This approach omits the risk of genetic manipulation for the host and is free from an extensive use of immunosuppressive drugs or expensive cell therapies. The systemic immunological unresponsiveness or hypo-responsiveness resulted from oral administration of

an antigen have been studied for over 50 years, and the promising outcomes showed immune anergy against several food allergens in human (79, 102, 103). In addition, this approach has been shown to be effective in decreasing autoimmune reactions in several autoimmune disorders such as experimental autoimmune encephalomyelitis, diabetes, and rheumatoid arthritis in animal models (104-106).

This also applied to induce a desirable tolerance in hemophilia A patient. A study on mucosal tolerance performed by Rawle et al. demonstrated that oral or nasal administration of the immunogenic FVIII C2 domain (FVIII-C2) in mice is able to relatively induce tolerance against this part even after encountering full-length FVIII (107).

In spite of the partial success in inducing tolerance, there was still the lack of a cost-effective technology to produce high amounts of FVIII antigens, and protect it against digestive enzymes in the stomach, which guaranteed proper delivery of the administered antigen to the immune system agents in the gut. Recent achievements in producing genetically engineered plants have helped to achieve human therapeutic proteins, biopharmaceuticals, and edible vaccines by using the chloroplast of crop plants (108, 109).

Furthermore, previous study showed that administration of frozen tobacco leaves in which C2 domain or the heavy chain of human BDD-FVIII were expressed. The result indicated a considerable reduction in inhibitor formation within two different strains of hemophilia A mice. Biological encapsulated antigens were orally administered twice per week and one month before starting traditional replacement therapy. It was shown that not only the allergic reactions against FIX in hemophilia B mice were abolished, but also pre-existing FVIII inhibitors increasingly were diminished in hemophilia A mice after using this method (110-112).

In fact, chloroplast genomic tools were developed

in order to determine the ribosomal locations which give rise to optimization of codon usage (109, 113). The major advantage of this application is that genetically engineered plants can express proteins which contain the antigen of interest, and in large amounts with a low price. This results in achieving therapeutic proteins potentially able to induce tolerance in oral administration (114).

Future perspectives in tolerogenic strategies for hemophilia A treatment

Different aspects of the inhibitor development in hemophilia A, and whether they might be

controlled are coming into focus. All developments in this area can lead to unleashing new approaches to practically treat or prevent hemophilia A complications.

One of the promising strategies for the treatment of genetic disorders *in utero* is maternal antigen transfer through which the antigen of interest is expressed to the immature immune system of the fetus, and antigen-specific tolerance may happen in the absence of pre-existing antibodies with high probability (115). It was shown that hemophilia A mice, which were intrave-

Table 1. Tolerance inducing protocols.

Tolerance protocol	Mechanism of action	Outcomes	References
Blockade of co-stimulatory pathways			
CTLA4-immunoglobulin (CTLA4-Ig)	Blockade of the B7/CD28 interaction	Prevention of the inhibitor formation in hemophilia A mice	50
CTLA4-Ig + anti-CD40L	Dual blockade of CD40/CD40L and B7/CD28 pathways	Long-term tolerance to FVIII induction in F8-plasmid treated hemophilia A mice	(51, 52)
T-cell depletion			
FVIII plasmid + Anti-CD3	Transforming growth factor- β levels and the generation of adaptive FVIII-specific Tregs	Long-term tolerance to FVIII	(54)
Treg induction			
IL-2/IL-2 mAb complexes	Rapid expansion of CD4 $^{+}$ CD25 $^{+}$ FOXP3 $^{+}$ Treg	Suppression of inhibitor formation to either FVIII replacement therapy or plasmid-mediated gene therapy of FVIII	(63-65)
Chimeric antigen receptor (CAR) gene transfer	FVIII-specific suppression by FOXP3 $^{+}$ Treg	Suppression of the antibody formation <i>in vitro</i> and <i>in vivo</i> in hemophilia A mice	(66)
B-cell depletion			
Anti-CD20 (Rituximab)	Depletion of the follicular B cells and increasing the regulatory T cells in the spleen	Remaining of the FVIII-specific hypo responsive state	(75)
Gene therapy			
Lentiviral vector-mediated gene therapy included miRNA target sequences (miRTs)	Restricting the expression of FVIII transgene to liver sinusoidal endothelial cells and myeloid cells	Exhibition of an average of 5–6% of normal FVIII activity, which is stable for up to 1 year after vector delivery	(98)
Oral tolerance induction			
Lettuce encapsulated clotting factor	Bioencapsulation and targeting of antigen to immune system, induction of Tregs	Acceleration of the decline of pre-existing FVIII inhibitors in hemophilia A mice	(109)

-nously injected by Fc fusions of FVIII A2 and C2 domains could successfully transfer the antigen to their fetus via the neonatal Fc receptor. Besides, after repeated administrations, an effective immune tolerance was also observed in the offspring of injected females in comparison with those that their mother had not been receiving antigen before (116, 117). These new achievements in maternal antigen transfer broaden our perspective about other strategies to develop antigen-specific regulatory T cells obtained from thymus or periphery. Although it would be a good strategy, a large number of antigens will be needed to achieve successful antigen transfer to a fetus that limits its application. In replacement therapy, development of Fc-conjugated FVIII has taken many efforts to produce more efficient FVIII with a greater half-life (118). Due to the importance of these molecules in the development of the immune tolerance, many attempts have focused on tolerogenic properties of Fc sequences (118). In the era of novel therapies, such as Emicizumab, a humanized monoclonal bispecific antibody that mimics the role of FVIII in the coagulation cascade and promotes thrombin generation, the management of hemophilia A patients who experience anaphylaxis to replacement therapy is becoming easier and may obviate the need for ITI.

During the last decade, a nanoparticle approach has been developed to provide an alternative to engineer cellular therapies for tolerance; these kinds of nanoparticles have also been used for drug delivery and vaccine development (119). Such nanoparticles can contain drugs, and are delivered with the target antigen and presumably are taken up by tolerogenic APCs, and induce Tregs (120, 121). The use of Rapamycin-containing nanoparticles for tolerance was successfully used by Zhang et al. for FVIII (122). Several other approaches, in addition to the above strategies, are being developed to induce tolerance to FVIII.

Conclusion

In conclusion, many experimental investigations have been performed to evaluate the effectiveness of inducing tolerogenic responses to overcome the main complication of hemophilia A patients, which is the anti FVIII inhibitor development (Table 1). Considering all the pros and cons, these approaches follow common destinations comprising cost-effectivity, durability, and time-efficiency of the treatment. Time will tell which of these approaches may become the best clinical therapy in the future.

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Conflict of Interest

There is no conflict of interest to declare.

References

1. Soucie JM, Evatt B, Jackson D. Occurrence of hemophilia in the United States. *Am J Hematol* 1998;59:288-94.
2. Weyand AC, Pipe SW. New therapies for hemophilia. *Blood* 2019;133:389-98.
3. Gouw SC, van den Berg HM, Fischer K, et al. Intensity of factor VIII treatment and inhibitor development in children with severe hemophilia A: the RODIN study. *Blood* 2013;121:4046-55.
4. Wight J, Paisley S. The epidemiology of inhibitors in haemophilia A: a systematic review. *Haemophilia* 2003;9:418-35.
5. Margaglione M, Intrieri M. Genetic Risk Factors and Inhibitor Development in Hemophilia: What Is Known and Searching for the Unknown. *Semin Thromb Hemost* 2018;44:509-16.
6. Hosseini S, Arabi S, Yari F, et al. HLA-DRB1* 01: 01, but not HLA-DRB1: 1503 or HLA-DRB1* 11, is associated with decreased inhibitor risk in Iranian hemophilia A patients. *Transfus Apher Sci* 2019;58:669-73.
7. Coppola A, Di Minno MN, Santagostino E. Optimizing management of immune tolerance induction in patients with severe haemophilia A and inhibitors: towards evidence-based approaches. *Br J Haematol* 2010;150:515-28.

8. Jimenez-Yuste V, Kruse-Jarres R, Rangarajan S, et al. The evolving role and future relevance of plasma-derived therapies in the management of bleeding disorders. *Thromb Haemost* 2016;116 Suppl 1:S1.
9. Carpenter SL, Khair K, Gringeri A, et al. Prophylactic bypassing agent use before and during immune tolerance induction in patients with haemophilia A and inhibitors to FVIII. *Haemophilia* 2018;24:570-7.
10. Napolitano M, Kessler CM. Hemophilia A and hemophilia B. *Consultative Hemostasis and Thrombosis*: Elsevier; 2019. p. 39-58.
11. Sherman A, Biswas M, Herzog RW. Tolerance induction in hemophilia: innovation and accomplishments. *Curr Opin Hematol* 2018;25:365-72.
12. Wight J, Paisley S, Knight C. Immune tolerance induction in patients with haemophilia A with inhibitors: a systematic review. *Haemophilia* 2003;9:436-63.
13. Valentino LA, Kempton CL, Kruse-Jarres R, et al. US Guidelines for immune tolerance induction in patients with haemophilia a and inhibitors. *Haemophilia* 2015;21:559-67.
14. Rocino A, Cortesi PA, Scalzone L, et al. Immune tolerance induction in patients with haemophilia a and inhibitors: effectiveness and cost analysis in an European Cohort (The ITER Study). *Haemophilia* 2016;22:96-102.
15. Gitschier J, Wood WI, Goralka TM, et al. Characterization of the human factor VIII gene. *Nature* 1984;312:326-30.
16. Randi AM, Smith KE, Castaman G. von Willebrand factor regulation of blood vessel formation. *Blood* 2018;132:132-40.
17. Kaufman RJ, Pipe SW. Regulation of factor VIII expression and activity by von Willebrand factor. *Thromb Haemost* 1999;82:201-8.
18. Metzner H, Schulte S, Weimer T. Covalent complex of von Willebrand Factor and factor VIII, compositions, and uses relating thereto. Google Patents; 2018.
19. Susree M, Anand M. Importance of Initial Concentration of Factor VIII in a Mechanistic Model of In Vitro Coagulation. *Acta Biotheor* 2018;66:201-12.
20. Toole JJ, Pittman DD, Orr EC, et al. A large region (approximately equal to 95 kDa) of human factor VIII is dispensable for in vitro procoagulant activity. *Proc Natl Acad Sci U S A* 1986;83:5939-42.
21. Batsuli G, Deng W, Healey JF, et al. High-affinity, noninhibitory pathogenic C1 domain antibodies are present in patients with hemophilia A and inhibitors. *Blood* 2016;128:2055-67.
22. Minno GD, Santagostino E, Pratt K, et al. New predictive approaches for ITI treatment. *Haemophilia* 2014;20 Suppl 6: 27-43.
23. Ibrahim UA, Ahmed SG. Determinants and modifiers of bleeding phenotypes in haemophilia-A: General and tropical perspectives. *Egypt J Med Hum Genet* 2018;19:171-8.
24. Fulcher CA, de Graaf Mahoney S, Roberts JR, et al. Localization of human factor FVIII inhibitor epitopes to two polypeptide fragments. *Proc Natl Acad Sci U S A* 1985;82: 7728-32.
25. Lacroix-Desmazes S, Bayry J, Misra N, et al. The prevalence of proteolytic antibodies against factor VIII in hemophilia A. *N Engl J Med* 2002;346:662-7.
26. Ghosh K, Shetty S. Immune response to FVIII in hemophilia A: an overview of risk factors. *Clin Rev Allergy Immunol* 2009;37:58-66.
27. van Helden PM, Van Haren SD, Fijnvandraat K, et al. Factor VIII-specific B cell responses in haemophilia A patients with inhibitors. *Haemophilia* 2010;16:35-43.
28. Scott DW, Pratt KP, Miao CH. Progress toward inducing immunologic tolerance to factor VIII. *Blood* 2013;121:4449-56.
29. Lacroix-Desmazes S, Navarrete AM, Andre S, et al. Dynamics of factor VIII interactions determine its immunologic fate in hemophilia A. *Blood* 2008;112:240-9.
30. Lovgren KM, Sondergaard H, Skov S, et al. Non-genetic risk factors in haemophilia A inhibitor management - the danger theory and the use of animal models. *Haemophilia* 2016;22: 657-66.
31. Tieu P, Chan A, Matino D. Molecular Mechanisms of Inhibitor Development in Hemophilia. *Mediterr J Hematol Infect Dis* 2020;12:e2020001.
32. Varthaman A, Lacroix-Desmazes S. Pathogenic immune response to therapeutic factor VIII: exacerbated response or failed induction of tolerance? *Haematologica* 2019;104:236-44.
33. Lacroix-Desmazes S, Voorberg J, Lillicrap D, et al. Tolerating Factor VIII: Recent Progress. *Front Immunol* 2019;10:2991.

34. Scott DW, Pratt KP. Factor VIII: Perspectives on Immunogenicity and Tolerogenic Strategies. *Front Immunol* 2019;10:3078.

35. Wroblewska A, Reipert BM, Pratt KP, et al. Dangerous liaisons: how the immune system deals with factor VIII. *J Thromb Haemost* 2013;11:47-55.

36. Peyvandi F, Mannucci PM, Garagiola I, et al. A Randomized Trial of Factor VIII and Neutralizing Antibodies in Hemophilia A. *N Engl J Med* 2016;374:2054-64.

37. Reipert BM. B-cell memory against factor VIII. *Cell Immunol* 2016;301:49-58.

38. Batsuli G, Ito J, Mercer R, et al. Anti-C1 domain antibodies that accelerate factor VIII clearance contribute to antibody pathogenicity in a murine hemophilia A model. *J Thromb Haemost* 2018;16:1779-88.

39. Qian J, Collins M, Sharpe AH, et al. Prevention and treatment of factor VIII inhibitors in murine hemophilia A. *Blood* 2000;95:1324-9.

40. Krishnamoorthy S, Liu T, Drager D, et al. Recombinant factor VIII Fc (rFVIIIFc) fusion protein reduces immunogenicity and induces tolerance in hemophilia A mice. *Cell Immunol* 2016;301:30-9.

41. Waters B, Lillicrap D. The molecular mechanisms of immunomodulation and tolerance induction to factor VIII. *J Thromb Haemost* 2009;7:1446-56.

42. Kreuz W. The role of VWF for the success of immune tolerance induction. *Thromb Res* 2008;122:S7-S12.

43. Koeberl DD. Immunomodulating gene therapy. *Google Patents*; 2015.

44. Lai JD, Lillicrap D. Factor VIII inhibitors: advances in basic and translational science. *Int J Lab Hematol* 2017;39:6-13.

45. De Groot AS, Moise L, McMurry JA, et al. Activation of natural regulatory T cells by IgG Fc-derived peptide "Tregitopes". *Blood* 2008;112:3303-11.

46. Algiman M, Dietrich G, Nydegger UE, et al. Natural antibodies to factor VIII (anti-hemophilic factor) in healthy individuals. *Proc Natl Acad Sci U S A* 1992;89:3795-9.

47. Hausl C, Ahmad RU, Sasgany M, et al. High-dose factor VIII inhibits factor VIII-specific memory B cells in hemophilia A with factor VIII inhibitors. *Blood* 2005;106:3415-22.

48. Qian J, Burkly LC, Smith EP, et al. Role of CD154 in the secondary immune response: the reduction of pre-existing splenic germinal centers and anti-factor VIII inhibitor titer. *Eur J Immunol* 2000;30:2548-54.

49. Nadler SG, Tamura JK, Price L, et al. Methods of treating immune diseases by administering antibody polypeptides that specifically bind CD40L. *Google Patents*; 2019.

50. Scott DW. Inhibitors - cellular aspects and novel approaches for tolerance. *Haemophilia* 2014;20 Suppl 4:80-6.

51. Rogers GL, Herzog RW. Gene therapy for hemophilia. *Front Biosci (Landmark Ed)* 2015;20:556-603.

52. Miao CH, Ye P, Thompson AR, et al. Immunomodulation of transgene responses following naked DNA transfer of human factor VIII into hemophilia A mice. *Blood* 2006;108:19-27.

53. Lai JD, Georgescu MT, Hough C, et al. To clear or to fear: An innate perspective on factor VIII immunity. *Cell Immunol* 2016;301:82-9.

54. Peng B, Ye P, Rawlings DJ, et al. Anti-CD3 antibodies modulate anti-factor VIII immune responses in hemophilia A mice after factor VIII plasmid-mediated gene therapy. *Blood* 2009;114:4373-82.

55. Herzog RW, Kuteyeva V, Saboungi R, et al. Reprogrammed CD4(+) T Cells That Express FoxP3(+) Control Inhibitory Antibody Formation in Hemophilia A Mice. *Front Immunol* 2019;10:274.

56. Wang X, Terhorst C, Herzog RW. In vivo induction of regulatory T cells for immune tolerance in hemophilia. *Cell Immunol* 2016;301:18-29.

57. Clemente-Casares X, Blanco J, Ambalavanan P, et al. Expanding antigen-specific regulatory networks to treat autoimmunity. *Nature* 2016;530:434-40.

58. Dobrzenski E, Fitzgerald JC, Cao O, et al. Prevention of cytotoxic T lymphocyte responses to factor IX-expressing hepatocytes by gene transfer-induced regulatory T cells. *Proc Natl Acad Sci U S A* 2006;103:4592-7.

59. Mingozi F, Hasbrouck NC, Basner-Tschakarjan E, et al. Modulation of tolerance to the transgene product in a nonhuman primate model of AAV-mediated gene transfer to liver. *Blood* 2007;110:2334-41.

60. Cao O, Dobrzenski E, Wang L, et al. Induction and role of regulatory CD4+ CD25+ T cells in tolerance to the transgene product following hepatic in vivo gene transfer. *Blood* 2007;110:1132-40.

61. Ohkura N, Kitagawa Y, Sakaguchi S. Development and

maintenance of regulatory T cells. *Immunity* 2013;38:414-23.

62. Nayak S, Cao O, Hoffman BE, et al. Prophylactic immune tolerance induced by changing the ratio of antigen-specific effector to regulatory T cells. *J Thromb Haemost* 2009;7:1523-32.

63. Webster KE, Walters S, Kohler RE, et al. In vivo expansion of T reg cells with IL-2-mAb complexes: induction of resistance to EAE and long-term acceptance of islet allografts without immunosuppression. *J Exp Med* 2009;206:751-60.

64. Liu CL, Ye P, Yen BC, et al. In vivo expansion of regulatory T cells with IL-2/IL-2 mAb complexes prevents anti-factor VIII immune responses in hemophilia A mice treated with factor VIII plasmid-mediated gene therapy. *Mol Ther* 2011;19:1511-20.

65. Liu CL, Ye P, Lin J, et al. Long-term tolerance to factor VIII is achieved by administration of interleukin-2/interleukin-2 monoclonal antibody complexes and low dosages of factor VIII. *J Thromb Haemost* 2014;12:921-31.

66. Kim YC, Zhang AH, Su Y, et al. Engineered antigen-specific human regulatory T cells: immunosuppression of FVIII-specific T- and B-cell responses. *Blood* 2015;125:1107-15.

67. Wang X, Riviere I. Clinical manufacturing of CAR T cells: foundation of a promising therapy. *Mol Ther Oncolytics* 2016;3:16015.

68. Levine BL, Miskin J, Wonnacott K, et al. Global Manufacturing of CAR T Cell Therapy. *Mol Ther Meth Clin Dev* 2017;4:92-101.

69. Gomes-Silva D, Ramos CA. Cancer Immunotherapy Using CAR-T Cells: From the Research Bench to the Assembly Line. *Biotechnol J* 2018;13.

70. Pan D, Buning H, Ling C. Rational Design of Gene Therapy Vectors. *Mol Ther Methods Clin Dev* 2019;12:246-7.

71. Yoon J, Schmidt A, Zhang AH, et al. FVIII-specific human chimeric antigen receptor T-regulatory cells suppress T- and B-cell responses to FVIII. *Blood* 2017;129:238-45.

72. Parvathaneni K, Abdeladhim M, Pratt KP, et al. Hemophilia A inhibitor treatment: the promise of engineered T-cell therapy. *Transl Res* 2017;187:44-52.

73. Bluestone JA, Tang Q. Treg cells-the next frontier of cell therapy. *Science* 2018;362:154-5.

74. Corti M, Elder M, Falk D, et al. B-Cell Depletion is Protective Against Anti-AAV Capsid Immune Response: A Human Subject Case Study. *Mol Ther Methods Clin Dev* 2014;1.

75. Zhang AH, Skupsky J, Scott DW. Effect of B-cell depletion using anti-CD20 therapy on inhibitory antibody formation to human FVIII in hemophilia A mice. *Blood* 2011;117:2223-6.

76. Biswas M, Rogers GL, Sherman A, et al. Combination therapy for inhibitor reversal in haemophilia A using monoclonal anti-CD20 and rapamycin. *Thromb Haemost* 2017;117:33-43.

77. Doshi BS, Arruda VR. Gene therapy for hemophilia: what does the future hold? *Ther Adv Hematol* 2018;9:273-93.

78. Nathwani AC, Reiss UM, Tuddenham EG, et al. Long-term safety and efficacy of factor IX gene therapy in hemophilia B. *N Engl J Med* 2014;371:1994-2004.

79. Sherman A, Biswas M, Herzog RW. Innovative Approaches for Immune Tolerance to Factor VIII in the Treatment of Hemophilia A. *Front Immunol* 2017;8:1604.

80. Annoni A, Gregori S, Naldini L, et al. Modulation of immune responses in lentiviral vector-mediated gene transfer. *Cell Immunol* 2019;342:103802.

81. Pickar AK, Gersbach CA. Gene therapies for hemophilia hit the mark in clinical trials. *Nat Med* 2018;24:121-2.

82. Nathwani A, McIntosh J, Tuddenham E, et al. Methods of delivering factor VIII encoding nucleic acid sequences. Google Patents; 2018.

83. Doering CB, Denning G, Shields JE, et al. Preclinical Development of a Hematopoietic Stem and Progenitor Cell Bioengineered Factor VIII Lentiviral Vector Gene Therapy for Hemophilia A. *Hum Gene Ther* 2018;29:1183-201.

84. Vetrini F, Ng P. Gene therapy with helper-dependent adenoviral vectors: current advances and future perspectives. *Viruses* 2010;2:1886-917.

85. Falkner F-G, Horling F, Lengler J, et al. Viral vectors encoding recombinant FVIII variants with increased expression for gene therapy of hemophilia A. Google Patents; 2019.

86. Matsui H, Shibata M, Brown B, et al. A murine model for induction of long-term immunologic tolerance to factor VIII does not require persistent detectable levels of plasma factor VIII and involves contributions from Foxp3+ T regulatory cells. *Blood* 2009;114:677-85.

87. Arruda VR. The search for the origin of factor VIII synthesis and its impact on therapeutic strategies for hemophilia A. *Haematologica* 2015;100:849-50.

88. Yadav N, Kanjirakkuzhiyil S, Ramakrishnan M, et al. Factor

VIII can be synthesized in hemophilia A mice liver by bone marrow progenitor cell-derived hepatocytes and sinusoidal endothelial cells. *Stem Cells Dev* 2012;21:110-20.

89. Sack BK, Herzog RW, Terhorst C, et al. Development of Gene Transfer for Induction of Antigen-specific Tolerance. *Mol Ther Methods Clin Dev* 2014;1:14013.

90. Mingozi F, Liu YL, Dobrzenski E, et al. Induction of immune tolerance to coagulation factor IX antigen by in vivo hepatic gene transfer. *J Clin Invest* 2003;111:1347-56.

91. Kumar SR, Markusic DM, Biswas M, et al. Clinical development of gene therapy: results and lessons from recent successes. *Mol Ther Methods Clin Dev* 2016;3:16034.

92. Agudo J, Ruzo A, Kitur K, et al. A TLR and non-TLR mediated innate response to lentiviruses restricts hepatocyte entry and can be ameliorated by pharmacological blockade. *Mol Ther* 2012;20:2257-67.

93. Alfranca A, Campanero MR, Redondo JM. New Methods for Disease Modeling Using Lentiviral Vectors. *Trends Mol Med* 2018;24:825-37.

94. Brown BD, Cantore A, Annoni A, et al. A microRNA-regulated lentiviral vector mediates stable correction of hemophilia B mice. *Blood* 2007;110:4144-52.

95. Cantore A, Ranzani M, Bartholomae CC, et al. Liver-directed lentiviral gene therapy in a dog model of hemophilia B. *Sci Transl Med* 2015;7:277ra28.

96. Borsotti C, Follenzi A. New technologies in gene therapy for inducing immune tolerance in hemophilia A. *Expert Rev Clin Immunol* 2018;14:1013-9.

97. Jobson J, Brown BD. Micromanaging Tolerance in Hemophilia A Gene Therapy. *Mol Ther* 2017;25:1739-40.

98. Merlin S, Cannizzo ES, Borroni E, et al. A Novel Platform for Immune Tolerance Induction in Hemophilia A Mice. *Mol Ther* 2017;25:1815-30.

99. Agudo J, Ruzo A, Tung N, et al. The miR-126-VEGFR2 axis controls the innate response to pathogen-associated nucleic acids. *Nat Immunol* 2014;15:54-62.

100. Wang X, Sherman A, Liao G, et al. Mechanism of oral tolerance induction to therapeutic proteins. *Adv Drug Deliv Rev* 2013;65:759-73.

101. Hussey Freeland DM, Fan-Minogue H, Spergel JM, et al. Advances in food allergy oral immunotherapy: toward tolerance. *Curr Opin Immunol* 2016;42:119-23.

102. Andorf S, Purington N, Block WM, et al. Anti-IgE treatment with oral immunotherapy in multifood allergic participants: a double-blind, randomised, controlled trial. *Lancet Gastroenterol Hepatol* 2018;3:85-94.

103. Chipps BE, Ciaccio CE, Rosén K, et al. Real-world attitudes among allergists/immunologists regarding oral immunotherapy and preferred terminology. *J Allergy Clin Immunol Pract* 2019;7:721-3. e9.

104. Weiner MD, Howard L. Oral tolerance for the treatment of autoimmune diseases. *Annu Rev Med* 1997;48:341-51.

105. Weiner HL, da Cunha AP, Quintana F, et al. Oral tolerance. *Immunol Rev* 2011;241:241-59.

106. Buerth C, Mausberg AK, Heininger MK, et al. Oral Tolerance Induction in Experimental Autoimmune Encephalomyelitis with *Candida utilis* Expressing the Immunogenic MOG35-55 Peptide. *PLoS One* 2016;11: e0155082.

107. Rawle FE, Pratt KP, Labelle A, et al. Induction of partial immune tolerance to factor VIII through prior mucosal exposure to the factor VIII C2 domain. *J Thromb Haemost* 2006;4:2172-9.

108. Daniell H. Expression of human interferon in transgenic chloroplasts. Google Patents; 2017.

109. Kwon KC, Sherman A, Chang WJ, et al. Expression and assembly of largest foreign protein in chloroplasts: oral delivery of human FVIII made in lettuce chloroplasts robustly suppresses inhibitor formation in haemophilia A mice. *Plant Biotechnol J* 2018;16:1148-60.

110. Sherman A, Su J, Lin S, et al. Suppression of inhibitor formation against FVIII in a murine model of hemophilia A by oral delivery of antigens bioencapsulated in plant cells. *Blood* 2014;124:1659-68.

111. Wang X, Su J, Sherman A, et al. Plant-based oral tolerance to hemophilia therapy employs a complex immune regulatory response including LAP+CD4+ T cells. *Blood* 2015;125: 2418-27.

112. Daniell H, Roland H. Administration of plant expressed oral tolerance agents. Google Patents; 2018.

113. Kwon KC, Chan HT, Leon IR, et al. Codon Optimization to Enhance Expression Yields Insights into Chloroplast Translation. *Plant Physiol* 2016;172:62-77.

114. Kwon KC, Daniell H. Low-cost oral delivery of protein drugs bioencapsulated in plant cells. *Plant Biotechnol J* 2015;13:

1017-22.

115. Sagar R, Götherström C, David AL, et al. Fetal stem cell transplantation and gene therapy. *Best Pract Res Clin Obstet Gynaecol* 2019.

116. Jennewein MF, Abu-Raya B, Jiang Y, et al. Transfer of maternal immunity and programming of the newborn immune system. *Semin Immunopathol* 2017;39:605-13.

117. Gupta N, Culina S, Meslier Y, et al. Regulation of immune responses to protein therapeutics by transplacental induction of T cell tolerance. *Sci Transl Med* 2015;7:275ra21.

118. Carcao M, Shapiro A, Staber JM, et al. Recombinant factor VIII Fc fusion protein for immune tolerance induction in patients with severe haemophilia A with inhibitors-A retrospective analysis. *Haemophilia* 2018;24:245-52.

119. Ilyinskii PO, Roy CJ, O'Neil CP, et al. Adjuvant-carrying synthetic vaccine particles augment the immune response to encapsulated antigen and exhibit strong local immune activation without inducing systemic cytokine release. *Vaccine* 2014;32:2882-95.

120. Maldonado RA, LaMothe RA, Ferrari JD, et al. Polymeric synthetic nanoparticles for the induction of antigen-specific immunological tolerance. *Proc Natl Acad Sci U S A* 2015;112:E156-65.

121. Getts DR, Shea LD, Miller SD, et al. Harnessing nanoparticles for immune modulation. *Trends Immunol* 2015;36:419-27.

122. Zhang AH, Rossi RJ, Yoon J, et al. Tolerogenic nanoparticles to induce immunologic tolerance: Prevention and reversal of FVIII inhibitor formation. *Cell Immunol* 2016;301:74-81.