

# Factor VIII Inhibitors: Risk Factors and Methods for Prevention and Immune Modulation

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**Abstract** Patients with hemophilia A are deficient in coagulation Factor VIII. This bleeding disorder can be treated with Factor VIII replacement therapy, but close to a third of patients will be immunized to the treatment and begin to form inhibitory antibodies known as “inhibitors”. These inhibitors will render the treatment ineffective and represent the most severe complication in the treatment of hemophilia A. In this review, we highlight factors involved in inhibitor development and emphasize research being done to modulate the immune response to this life-saving therapy.

**Keywords** Hemophilia inhibitors · Immunogenicity · Immune tolerance · FVIII

## Introduction

Hemophilia A is a bleeding disorder defined by a functional deficiency in clotting Factor VIII (FVIII), a large glycoprotein with multiple domains in the order NH<sub>2</sub>-A1-A2-B-A3-C1-C2-COOH. The corresponding gene spans 180 kb and constitutes 0.1% of the X chromosome [1]. It is primarily synthesized in the liver and circulates as a heterodimer in a complex with the much larger protein, von Willebrand factor (vWF), which protects it from degradation and endocytosis, and concentrates it at the site of action [1–3]. In its active form, FVIII functions as a regulatory co-factor that anchors activated factor IX and factor X, a serine protease, to platelet phospholipids forming

the “Xase” complex and increasing the rate of FX activation nearly 200,000-fold [4]. FVIII plays an essential role in the intrinsic clotting pathway, but only a low concentration (0.2 µg/ml plasma) is needed to ensure proper function. When the plasma concentration of FVIII is insufficient, bleeding episodes will occur that are characterized by increased duration rather than increased intensity [1, 2].

The severity of hemophilia A varies depending on the nature of the mutation to FVIII and activity of any endogenous protein that may have formed. About two thirds of patients are designated as severely affected because they have less than 1% functional FVIII. Those with moderate disease have activity levels between 1% and 5%, whereas mild disease is defined as 5–50% of normal FVIII function. Each of these reflects a spectrum of mutations that lead to a loss of part or all of the FVIII protein. The *FVIII* gene itself is prone to mutation. Indeed, about 30% of the one in 5,000 males affected have no family history.

Hemophilia A leads to a clinically heterogeneous set of bleeding problems, or diatheses, following surgery or trauma. Trauma, in particular, occurs early in life with bleeding into the muscle and joints (primarily the knees, elbows, ankles, shoulders, and hips). In a vicious cycle, trauma will precipitate joint problems that are followed by inflammation and synovitis. This leads to more bleeding into the joints, ultimately resulting in disability or even death from excess blood loss. To treat bleeding episodes, physicians use recombinant or plasma-derived FVIII. Depending on the severity of the disease and mutation involved, patients can develop a deleterious response to the treatment. Thus, their immune system identifies this protein as foreign and mounts a humoral response to epitopes in FVIII. A subset of these antibodies block the functional regions and are termed “inhibitors.” These inhibitory antibodies are typically measured in a Bethesda assay [1, 5]. The factors that govern inhibitor formation and its modulation are the focus of this review.

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Inhibitor development is currently the most significant complication in the treatment of hemophilia. Although antibodies can bind various parts of the FVIII protein, inhibitors are primarily directed towards the A2, A3, and C2 domains where they function through steric hindrance preventing association with Factor IX and Factor X to form the Xase complex, binding of FVIII to vWF, and the binding of FVIII with phospholipid membranes [5]. Additional inhibitory antibodies may bind neo-epitopes formed when FVIII and vWF are bound and prevent normal dissociation of the complex, or they may inhibit proteolytic cleavage by thrombin. Moreover, the epitopes recognized may change over time and titers may decline over time without any change in treatment [6]. It has also been shown that anti-FVIII antibodies may possess enzymatic activity to hydrolyze FVIII [7, 8], and it has been proposed that immunoglobulins may accelerate the clearance of FVIII from circulation [9]. Understanding the factors that govern the formation of inhibitors and how they can be controlled is fundamental to providing adequate care to those affected.

As mentioned, treatment can have the unintended consequence of immunizing the patient to the cure. This may be unexpected considering the treatment is administered intravenously (i.v.), a route that is more tolerogenic than intramuscular or intraperitoneal delivery, and in the absence of any obvious adjuvant to produce “danger signals” [10]. Still, up to a third of all patients will respond with inhibitory antibodies. Risk factors that contribute to a patient’s response to this life-saving treatment have been excellently reviewed in the past [11] and have been updated in detail for this issue (Ghosh K and Shetty S). In this review, we will highlight a few of the factors involved in inhibitor development and emphasize research being done to modulate the immune response.

### **Type of mutation in the *FVIII* gene can affect immunogenicity**

The human *FVIII* gene is located at the end of the long arm of the X chromosome, spans 186 kb in length, and consists of 26 exons ranging in size from 69 to 3,106 bp [12]. Since the *FVIII* gene was cloned in 1984 [12–14], a large number of disease-causing mutations within this gene have been identified. A database of mutations in the factor VIII gene (HAMSTeRS) has been published in 1996 and regularly updated [15].

The type of genetic mutation in the *FVIII* gene clearly has a major influence on inhibitor formation [15–17]. Generally, mutations that result in absence or severe truncation of the FVIII protein are associated with the highest incidence of inhibitor formation [18–25]. These types of mutations include intron 22 inversion, intron 1

inversion, large deletions, and nonsense mutations [22]. Interestingly, even a single point mutation can result in inhibitor formation against the wild type of FVIII protein.

One reason for the high incidence of inhibitor formation in patients with mutations that resulted in the absence or severe truncation of FVIII is that these patients have failed to achieve either central or peripheral self-tolerance. The primary mechanism of central self-tolerance is mediated through the deletion of auto-reactive T cells. During their maturation in the thymus, immature T lymphocytes with high affinity to self-antigens are negatively selected through apoptosis. If self-reactive cells survive and enter the circulation, tolerance would need to be maintained in the periphery through the induction of anergy from the lack of co-stimulation or by regulatory T cells. In hemophilia A patients, the absence of peptides derived from endogenous FVIII protein prevents the deletion of antigen-specific T cells. Therefore, the infused FVIII would be perceived as a foreign protein, and the adaptive immune response could be triggered. The incidence of inhibitor formation would be predicted to be lower in those patients whose *FVIII* gene mutation still permits certain amount of FVIII antigen to be existed in the circulation. This appears to be the case (see below).

The presence of FVIII protein or even peptides derived from a mutant *FVIII* gene should mitigate inhibitor formation. One example is the relatively low incidence of inhibitor formation (0–16%) in patients with small deletion/insertion mutation [25, 26]. This type of mutation often leads to a frame shift and a subsequent stop codon, which would suggest an inhibitor incidence in the same range as nonsense mutations. However, a study conducted by Young et al. [27] revealed that, in one patient with a frameshift mutation in exon 14 (delT from A8TA2 at codon 1441, resulting in A10), the reading frame was apparently restored in 5% of the mRNA. The result was a moderate amount of functional, in-frame (A8 and A11) *FVIII* mRNA. This phenomenon was later confirmed by Oldenburg et al. [28], who demonstrated that FVIII antigen was detectable, using a highly sensitive ELISA protocol, in two of four patients with deletion or insertion mutations which caused a frame shift in exon 14, but none of the five patients with other nonsense or inversion mutation had detectable FVIII antigen.

Mis-sense mutations represent the major mutation type in mild/moderate hemophilia A and are usually associated with the presence of endogenous but functionally altered protein. Patients in this category have a lower risk for the formation of inhibitors (5%) [25]. Although functionally altered, endogenous FVIII would be sufficient to induce partial immune tolerance. However, there are exceptions. Studies by Hay [29] demonstrated that, within this group, there might be few high-risk FVIII genotypes clustered in

“hot spots” within the A2 and C2 domains, especially the Arg<sup>593</sup>-Cys and the Trp<sup>2229</sup>-Cys mutations, respectively. Up to 40% of the patients with point mutations located in the above sites developed inhibitors. An alternative mechanism may underlie inhibitor development in these patients. One possible explanation is that a *stable conformational change* might be introduced by mutation in those sites, which renders the mutant antigenically distinct from wild-type FVIII [29].

### Hereditary risk factors: role of HLA and SNPs

Considering the important role of MHC I and MHC II in antigen presentation, polymorphisms in these genes have long been suspected to play a role in the inhibitor formation in hemophilia A patients [30, 31]. However, strong evidence for this has not yet been found. The HLA class I alleles A3, B7, and C7 and the class II alleles DQA0102, DQB0602, and DR1501 were found more often in hemophilia A patients with inhibitors [32, 33]. But the correlation was weak, and such a trend was not seen again in the more recent Malmö International Brother Study (MIBS) [34], in which sibling hemophilia A patients were enrolled in the cohort.

Interestingly, a strong link between TNF- $\alpha$  gene polymorphism and the inhibitor formation in hemophilia A siblings was found in the MIBS study [34]. Among the 164 hemophilia A siblings studied, homozygosity of -308 A allele was identified in 22 individuals (13.4%), 16 of which developed inhibitors (72.7%). This single nucleotide polymorphism (SNP) in the promoter region of the TNF- $\alpha$  is associated with increased production and secretion of TNF- $\alpha$  in patients with inflammatory bowel diseases and myasthenia gravis [35, 36]. It is also noteworthy that the TNF- $\alpha$  gene locates within the class III of MHC. Whether this TNF- $\alpha$  SNP is an independent risk factor for inhibitor formation may require further investigation with larger cohorts.

Recently, *IL10G*, an allele with 134 bp in one of the CA repeat microsatellites located in the promoter region of the *IL10* gene, has been shown to be a risk factor for inhibitor formation in hemophilia A patients. IL-10 is an important immunoregulatory cytokine, and the *IL10G* allele has been reported to associate with high antibody production in autoimmune diseases, like Wegener's granulomatosis and myasthenia gravis [37, 38]. The MIBS study [39] demonstrated a strong correlation between the *IL10G* allele and inhibitor formation in siblings with hemophilia A. This allele was identified in 44 of 164 (26.8%) patients with hemophilia A, and 32 of them (72.7%) developed inhibitors. However, the exact mechanism by which this IL-10 polymorphism increases the risk of inhibitor formation remains to be elucidated.

### Environmental risk factors

The discordance of the monozygotic twins in inhibitor formation reported in the MIBS study [40] suggested that, in addition to the decisive role of genetic predisposition, environmental risk factors also have a major role to play. Some of the environmental risk factors appear to be the age of first exposure, the mode of administration, ongoing infection, and probably vaccination.

Studies have indicated the age of first exposure to FVIII to be a risk factor for inhibitor formation [41–42]. However, in these studies, the type of FVIII mutation was not controlled, and the need for earlier treatment might reflect a more severe disease phenotype or importantly more intensive therapy for major bleeds. Indeed, in the study by Santagostino et al. [43], when the genetic factors were adjusted, such association was not seen in a cohort of 108 hemophilia A children who received FVIII treatment. Interestingly, in a subgroup of 25 cases, patients who started early prophylaxis had a lower inhibitor risk than those treated on demand. Therefore, there is a need to further explore if the age of first exposure has a true impact on inhibitor formation.

The mode of administration, e.g., a bolus injection vs. continuous infusion, could be another risk factor, with continuous infusion appearing more likely to lead to inhibitor formation in many hemophilia A patients [44–47]. However, the circumstances, which lead to the choice of administration method, also have implications for interpretation of risk. Therefore further, large sample size studies which control for intensity of treatment and bleeds are needed to delineate the risk of continuous infusion.

Whether ongoing infection or vaccination during the FVIII replacement therapy is a risk factor for inhibitor formation is still a largely open question. Such situations might be able to provide a “danger signal” to the patient's immune system and favor an effective immune response to FVIII, as in a mechanism proposed by Reipert et al. [48].

From an immunologic point of view, early intervention with prophylaxis should be more tolerogenic. However, humans are quite immunologically competent for most antigens even before birth, so other risk factors cited above must play a role. It is likely prophylaxis done under controlled conditions may be highly preferable to favor tolerance and avoid inhibitor formation.

### Nature of the product used in therapy

The type of anti-hemophilic product used (recombinant or plasma-derived) has triggered a debate in this field since the introduction of recombinant FVIII product. Experts disagree over whether or not new recombinant products create neo-antigens or lack protective chaperones, like vWF, and

may be more immunogenic [49, 50]. Now that viral inactivation techniques have virtually eliminated the threat for transmission of HIV or HCV, plasma-derived concentrates are safe and effective. However, the recombinant alternatives may offer a cheaper and readily available substitute. Key to the debate is the role of vWF, which is present in plasma-derived preparations but absent from the recombinant form. As mentioned, vWF serves to stabilize and concentrate FVIII at the site of action; it also covers regions of the proteins where inhibitors can bind. However, recombinant FVIII protein should theoretically bind with high affinity to endogenous, circulating vWF. Thus, it is not immediately clear that this should make a significant difference.

To address these questions in a clinical setting, researchers have performed a number of retrospective and prospective studies, though there is still a need for randomized control trials large enough to withstand the rigors of evidence-based medicine [51]. In 2006, Goudemand et al. [52] published an analysis comparing responses to plasma-derived (pdFVIII), or recombinant FVIII (rFVIII) in 148 PUPs who were treated in 24 French hemophilic centers. They found that treatment with recombinant FVIII carries a 2.5- to 3-fold higher risk for the development of inhibitors. Additionally, non-whites were 3.5 to 6.7 times as likely to form inhibitors, and with a positive family history, patients are approximately 6-fold as likely to form inhibitors when treated with recombinant products. The authors suggest that a likely reason for a decreased response to pdFVIII is co-precipitation of immunomodulatory molecules like TGF $\beta$  and protective molecules like vWF, both of which are likely to help prevent initiation of inhibitors. However, this study does not conclusively end the debate on this topic and future studies may yield different data because the three recombinant products used are no longer on the market as “improved” products are continually becoming available.

The researchers in the CANAL cohort study [42] performed a similar trial, but reached different conclusions. They compared various FVIII treatments in 316 patients with severe hemophilia A born between 1990 and 2000. Overall, they found no significant difference in inhibitor formation between patients treated with recombinant or plasma-derived products and patients that changed products during treatment. Additionally, even though patients treated with a B Domain Deleted rFVIII were 40% more likely to form inhibitors when compared to those treated with full-length FVIII (RR, 1.4; CI 0.8–2.6), that difference was still not statistically significant. The authors note the discrepancy between their data and Goudemand’s published a year earlier. They suggest that the plasma-derived FVIII used in the earlier study was less immunogenic, and some of the 23 different plasma-derived products used in the more recent study may be more immunogenic.

Calvez et al. [53] have recently tried to unify these discrepancies by re-examining the data from four different retrospective studies comparing plasma-derived and recombinant FVIII [42, 52, 54, 55]. However, because the data were analyzed differently between groups, it is not possible to calculate a total risk between all the patients involved. Despite that obstacle, they are able to present the data from all the studies in a figure, which clearly indicates that, although not significant, there is an obvious trend indicating that recombinant products are more immunogenic.

It is important to consider that while retrospective analysis may provide evidence for one hypothesis over another, we may be unintentionally comparing apples and oranges [56]. Not all conclusions are based on the same end points (e.g., short-term vs. long-term titers), not all plasma-derived products have the same purity, not all recombinant products go through identical manufacturing steps, and the studies cannot be truly matched for patient risk factors. Additionally, in a hypothetical experiment in which 15 of 60 participants form inhibitors, some may publish a 25% incidence, but it should be noted that the 95% confidence interval ranges from 14.7% to 37.9% [56]. An official recommendation was published in 1999, stating that previously treated patients (PTPs) with >150 exposure days are considered tolerant to plasma-derived FVIII and are superior to PUPs to study inhibitor formation [6]. PUPs may respond to either plasma-derived or recombinant FVIII if they are not tolerant, while PTPs will only respond to neo-antigens if they are present on the newer products. This is important to consider when developing future trials.

Obviously, a less immunogenic FVIII product would also be desirable. In this context, two approaches are worth describing. Dasgupta et al. [57] recently reported that uptake of FVIII by dendritic cells depends in part on recognition of mannose residues. Indeed, demannosylated FVIII was not able to stimulate a human T cell clone *in vitro* [57]. This suggests that a functional demannosylated FVIII might be a less immunogenic product. Another approach would be to mutate FVIII residues that are required to anchor into MHC II grooves. This process is called “de-immunization” and has been utilized with a number of biotherapeutics, including monoclonal antibodies that were immunogenic [58]. Such a product, if it still maintained clotting activity, should theoretically be totally non-immunogenic. However, both of these approaches would lead to a non-tolerogenic product because they could not be presented to the immune system (see below).

#### **Other factors affecting inhibitor formation: aggregation and clotting as “danger” signals**

In addition to previously mentioned risk factors, immunogenicity may be altered due to aggregate formation from

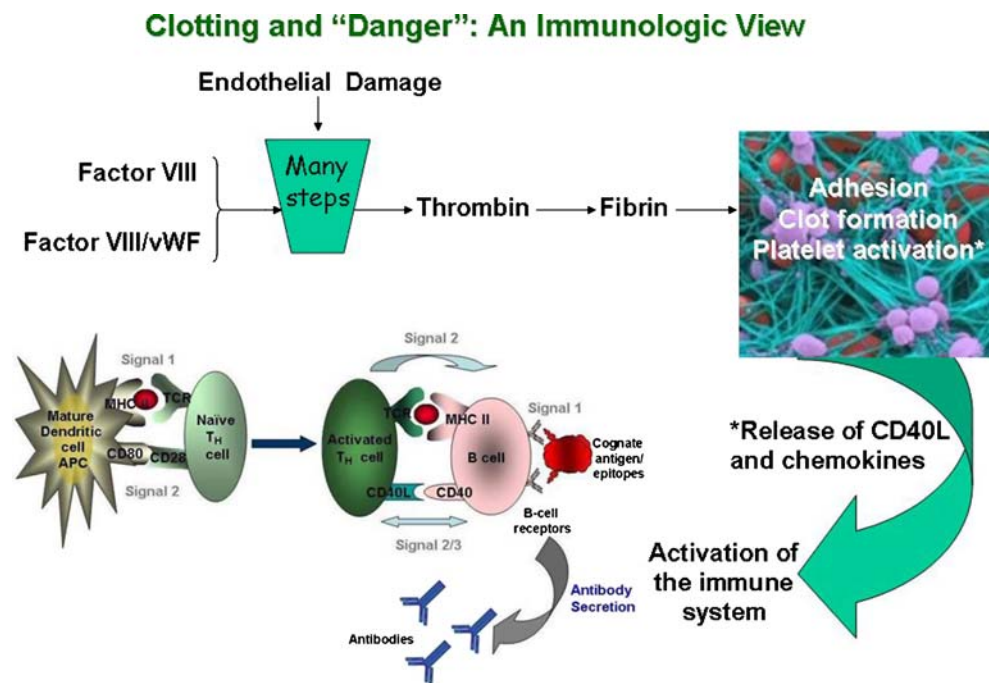
prolonged contact with plastic infusion devices. Aggregation is common with recombinant proteins and the exact nature will likely vary between expression systems and methods for preservation and storage. However, aggregation could account for a “danger” signal necessary to activate B lymphocytes. In one classic case, a change in product packaging led to significant inhibitor formation due to leachates (see ref. [58]). To study the effects of aggregation in the murine model for hemophilia A, Purohit et al. heated rFVIII to 80°C for 2 min and injected mice subcutaneously. The authors surprisingly found that heat-aggregated FVIII elicited a weaker immune response than the native protein [59]. It is necessary to note that the way they have induced aggregation may not entirely mimic aggregates in therapeutic concentrates, and the route of administration was not physiologic. Indeed, they also may have induced antibodies to neo-epitopes that do not have inhibitory properties. The study would have been enhanced by exploring the function of the protein used in the experiments.

It is well known that heating diminishes FVIII clotting activity. In our hands, heat treatment to 56°C for 30 min will totally inactivate FVIII as measured in a chromogenic assay, and causes significant unfolding of the protein and loss of several B cell epitopes. When heated FVIII was compared to native FVIII, we found that the latter was far more immunogenic (Skupsky et al. in preparation). This led us to suggest that clotting may provide one of the “danger” signals in the immunogenicity of FVIII (see Fig. 1). This provides a testable link between function and immunogenicity. The formation of a clot is not an inert event to the immune system. Platelets are aggregated and activated,

releasing chemokines and CD40 ligand, a potent co-stimulator of the immune system. This may act *locally* to promote stimulatory signals to the immune system, providing evidence once again that ‘location’ is most important in many endeavors!

With the exception of the clotting/danger hypothesis, efforts to establish that FVIII has intrinsic immunogenicity to activate the innate immune system have generally failed. Pfistershammer et al. [60] were unable to demonstrate the so-called *danger* signals for human monocyte-derived dendritic cells (DCs). These workers queried whether FVIII alone or complexed with von Willebrand factor (vWF) or even thrombin-activated FVIII would upregulate co-stimulatory molecules (such as CD80, CD86, or CD40) or MHC class II. They also did not find that FVIII could stimulate the expression of pro-inflammatory cytokines by DCs nor enhance the stimulatory ability of allogeneic DCs in a mixed lymphocyte culture system [60]. We also tested whether FVIII would provide any innate signals that would mimic Toll-like receptor signaling in the immune system. We examined whether FVIII stimulated murine B cells in vitro to enlarge, proliferate, or increase expression of class II and CD80/CD86 with negative results (I. Carey et al., unpublished). No obvious changes were observed in the spleens of mice injected i.v. with FVIII, although neither were full kinetics followed nor the local milieu of the splenic follicles examined. Recently, we found that when FVIII was co-administered with purified ovalbumin (OVA), the anti-OVA response was enhanced (Skupsky et al. in preparation). Perhaps, the presence of FVIII’s intrinsic immunogenicity was able to provide some “danger” signals for the OVA-reactive cells to

**Fig. 1** Clotting and danger: an immunologic view. The process of clotting, in which FVIII is a major co-factor, ultimately leads to thrombin cleavage, fibrin clots, and both platelet adhesion and activation. The latter results in the release of chemokines and potent immune co-stimulatory molecules like CD40L. These both recruit and activate antigen-presenting dendritic cells and lymphocytes to stimulate help for antibody formation



be primed. The nature of these signals remains to be determined. It is noteworthy that we and others have found that human FVIII is immunogenic even in hemostatically normal mice and in rhesus macaques [61, 62]. Thus, even in hosts that should be at least partially tolerant to FVIII, this protein has significant immunogenicity. The inherent immunogenicity of FVIII is an important obstacle to overcome in designing therapies to modulate inhibitor formation.

### Methods for prevention and immune modulation of inhibitor formation

FVIII not only functions at the site of vascular insult but also interacts with the vast network of lymphoid tissue throughout the body to engender humoral and cellular responses. Lacroix-Desmazes et al. [9] proposed that the immune response to FVIII is initiated in two locations. After i.v. injection, FVIII is carried to the spleen as are most blood-borne pathogens. It will also be loaded on APCs and carried to the draining lymph node from the site of injury (e.g., joints suffering from chronic inflammation) where co-stimulation may be derived from chronic inflammation [9]. The net result, depending on the factors cited above, is that an immune response to FVIII ensues with the formation of inhibitory antibodies that block the clotting activity of FVIII (and obviate the very therapy they need). Thus, an important goal is to reverse or preferably prevent inhibitor formation in patients receiving FVIII therapy.

Clearly, patients would prefer a cure to improved treatments for hemophilia. Although gene therapy is still several years from becoming a clinical reality, hemophilia may one day be treated by correction or replacement of the defective gene. Recent progress in this field has been reviewed extensively [63]. Hemophilia is an attractive disease for gene therapy because the therapeutic window is so wide; raising levels to between 3% and 10% is an acceptable endpoint. Additionally, the gene can be manipulated to improve stability [64] and it can be engineered to improve secretion [65]. There are a number of options for a target tissue, which can secrete FVIII including stem cells, platelets, and long-lived differentiated cells, including muscle and cells in the liver. Phase 1 clinical trials to introduce a functional FVIII or FIX gene have been attempted by i.v. infusion of a retroviral vector [66], by ex vivo transfection of plasmid DNA to autologous fibroblasts [67], by AAV-mediated gene transfer to skeletal muscle [68], and by AAV-mediated gene transfer to the liver [69]. All of these studies initially produced promising results, but ultimately failed to express therapeutic protein for a long period of time, often because of the immune response to the vector or the FVIII per se.

Tissue transplantation may provide another route to cure hemophilia A. A recent report [70] describes transplantation

of liver sinusoidal endothelial cells from a healthy donor mouse to a hemophilic FVIII knockout recipient. Injection of  $2 \times 10^6$  cells (10% of the endothelial compartments) through the portal vein led to engraftment of approximately  $2 \times 10^5$  cells, which raised FVIII to therapeutic levels at least 60 days post-transplant. Liver transplantation has previously been reported effective in hemophiliacs who qualify for surgery [71, 72], but surgery is always dangerous in patients with bleeding disorders and liver cell transplantation offers a less invasive alternative. Furthermore, one donor could treat several recipients, or this cell type could be used with the gene therapy in an autologous transplant. However, transplantation of allogeneic tissue would require immunosuppression and there still would be allo-recognition of the secreted FVIII, the major obstacle!

We suggest that attempts to induce tolerance to FVIII domains should be a primary goal of hemophilia A therapy in order to prevent the immune response to FVIII delivered either as a gene therapy expressed protein or a biotherapeutic. Thus, to use a baseball analogy, the goal should be to hit a few singles and a double (tolerance to FVIII domains) to score a run, rather than swing for the fences and try to hit a home run with gene therapy expression (and engender an immune response that puts you “out at home”).

A number of methods have been described that can lead to avoidance or modulation of the immune response to FVIII, and even tolerance in experimental animals with ongoing inhibitor formation. The former approach involves synthesis of recombinant FVIII with substitution of functional non-human FVIII, such as porcine FVIII (pFVIII), based on the observation that inhibitors often do not cross-react with pFVIII [73]. Thus, full-length plasma-derived pFVIII has been used to treat patients, including those with acquired hemophilia that are no longer responsive to the standard of care. Indeed, prior to the 1990s, when there was a practical concern for the transmission of HIV or hepatitis virus through blood-derived products, pFVIII was preferred because of its excellent safety record [74, 75]. Currently, the use of porcine-derived FVIII is focused on the use of A2-domain substituted FVIII especially in patients with anti-A2 inhibitors [73]. However, some patients may eventually produce antibodies that cross-react with these products.

Typically, when a patient presents with a significant inhibitor titer, high dose immune tolerance induction (ITI) is initiated. ITI was first reported in 1977 [76] and is currently the most accepted way to treat patients with inhibitors [77]. Most treatments involved high dose infusions (50–200 IU/kg) until Bethesda titers have been reduced or antibodies have entirely disappeared. Because not all patients are responsive to ITI, other adjunct treatments have been examined. Rituximab, an investigational chimeric antibody specific for human CD20 on B

cells, has been effective in difficult to treat patients [78] and may reduce the time and cost in treatable patients. Rituximab effectively removes most B cells from circulation, but fails to affect long-lived plasma cells that are CD20<sup>negative</sup>. Nonetheless, as an adjunct therapy, anti-CD20 treatment may have long-term benefits.

An alternative method to modulate immune responses to FVIII is to block co-stimulation either with anti-CD40L (CD154) or CTLA4-Ig. In the former case, significant ablation of germinal center formation after FVIII injection occurred, with the consequent elimination of anti-FVIII antibody production after FVIII [79]. However, this did not lead to tolerance induction. Because anti-CD154 can react with platelets, leading to thrombotic events, this approach is currently not recommended.

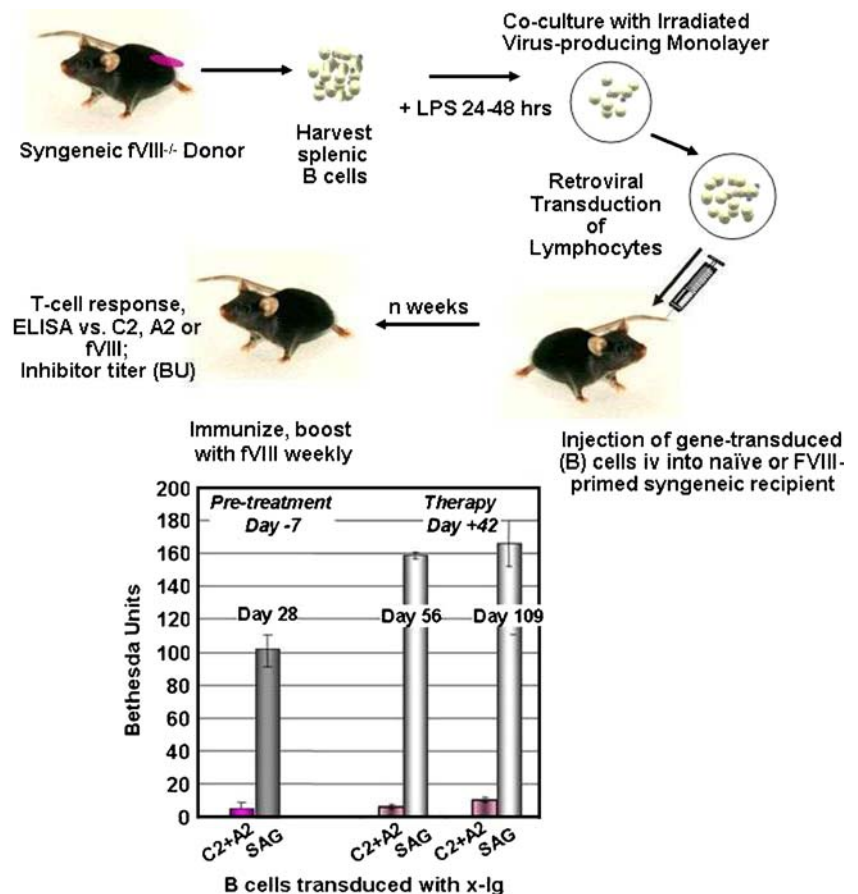
More promising is the use of CTLA4-Ig [80]. CTLA-4 is a surface protein on activated T cells and regulatory cells and it has higher affinity for B7 molecules than does CD28. Since CD28/B7 interactions are necessary for B cell activation and antibody production, blockade from CTLA-4 binding to B7 will inhibit co-stimulation and reduce both B and T cell activation. In the mouse model for hemophilia A, the immune response following i.v. delivery of FVIII is absent in mice that also lack B7.2. Furthermore, the recombinant CTLA4-Ig fusion protein will block this

interaction in normal mice and prevent both primary and secondary anti-FVIII responses [81].

Recently, Miao and colleagues [82] used a combination of immunosuppressive or immunomodulatory therapies to prevent inhibitor formation after naked DNA (encoding FVIII) transfer. These included cyclosporine A (CSA), rapamycin (RAP), mycophenylate mofetil (MMF), a combination of CSA and MMF, a combination of RAP and MMF, anti-CD154, recombinant murine CTLA4-Ig, and combination of anti-CD154 and CTLA4-Ig. Although some drugs led to suppression of immune responses in many animals, this effect was short lived. Interestingly, they found that the most effective was the combination of anti-CD154 and CTLA4-Ig, which prevented inhibitor formation and led to long-term tolerance. Most recently, this group found that short-term therapy with a monoclonal antibody to block another co-stimulatory pathway, i.e., ICOS/ICOSL, led to tolerance to human FVIII in hemophilia A mice treated with a *hFVIII* plasmid, thus allowing high-level FVIII functional activity for months, a very promising result with important implications for both gene therapy and tolerance induction [83].

Gene therapy to induce tolerance to FVIII is another important approach [84]. The focus of our lab has been to take advantage of the tolerogenic properties of IgG and B

**Fig. 2** Gene therapy for tolerance to FVIII in immunized hemophilia mice. B cells are activated and then transduced with a retroviral vectors encoding C2 fused to an IgG heavy chain and A2 fused to an IgG heavy chain. Recipients of these transduced B cells are rendered tolerant to further challenge with FVIII in terms of T cell proliferation, antibody formation to these domains and to whole FVIII, and importantly, inhibitor formation (*inset*). This has been successful in both naïve and in immunized recipients. Data modified from Lei and Scott [84]



cell presentation to create a platform for gene therapy for tolerance in many autoimmune diseases and hemophilia A. Success has been achieved with 12 different antigens in different mouse strains and in rats. For pre-clinical testing in hemophilia, we decided to target the C2 and A2 domains since most inhibitory antibodies are directed at epitopes in these important functional regions of FVIII. Therefore, we inserted the coding regions for residues S2173–Y2332 of the human FVIII C2 domain and S373–R740 of the FVIII A2 domain onto a mouse IgG heavy chain backbone, respectively, to create a retroviral vector [84]. B cells transduced with these vectors were injected into hemophilic mice. We then demonstrated that specific tolerance to each domain was induced by this protocol. Moreover, a combination of A2-IgG and C2-IgG expressing B cells induced tolerance to the full-length FVIII molecule, a result which supports the dominance of these domains in the immune response to FVIII, and that multiple constructs can be used for more complete tolerance coverage. Treatment was successful in primed mice and lasted over 100 days. Importantly, this therapy reduced inhibitor titers in naïve and in primed hemophilia A mice [84] (see Fig. 2).

This B cell-delivered gene therapy protocol for tolerance required MHC class II and B7 expression on the transduced B cells [85, 86], and was dependent on transfer to the endosomes and processing in a gamma interferon inducible lysosomal thiol reductase-dependent fashion [87]. Moreover, tolerance induction was dependent on CD25+ regulatory cells [84]. We are currently exploring the role of these regulatory T cells in this system using a fully backcrossed hemophilic mouse with a FoxP3–GFP fusion (Skupsky, to be published). Because tolerogenic B cells interact with regulatory T cells, which may go on to mediate tolerance, one could imagine a system where tolerogenic B cells are reintroduced to patients long enough to upregulate these antigen-specific T-regs and then deleted using an incorporated suicide gene. Such a system could affect long-term tolerance while avoiding the fear of insertional mutagenesis that is currently associated with other types of gene therapy. Proof of principle, that this technique will potentially be useful in patients, was recently obtained in our lab using a T cell clone (provided by Drs. Kate Pratt and Ruth Ettinger, Puget Sound Blood Center, Seattle). We transduced HLA-matched B cells with a C2-Ig construct and cultured the hemophilic T cell clone with these B cells. Such cells were no longer able to produce interferon gamma when subsequently challenged, whereas cell exposed to non-transduced B cells could (Scott et al., in preparation). The mechanisms involved need to be fully explored, but these data support the notion that gene therapy for tolerance may be applicable in the not-too-distant future for the treatment of hemophilia A inhibitors.

## Conclusion

Overall, there are multiple mechanisms linking inhibitor formation and the immunogenicity of FVIII. As we try to decipher underlying mechanisms of immunogenicity, we should be focused on novel products and approaches to modulate the immune response to FVIII in hemophilia A patients.

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