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To clear or to fear: An innate perspective on factor VIII immunity

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**A B S T R A C T**

The enigma that is factor VIII immunogenicity remains ever pertinent in the treatment of hemophilia A. Development of neutralizing antibodies against the therapeutic protein in 25–30% of patients likely depends on the appropriate activation of the innate immune response shortly following antigen encounter. Our understanding of this important immunological synapse remains ill-defined. In this review, we examine the three distinct factors contributing to the fate of factor VIII almost immediately after infusion: the characteristics of the protein, the cell, and the microenvironment. We propose a continuum between clearance and antigen presentation that facilitates removal of FVIII from circulation leading to either tolerance or immunity.

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1. Introduction

Mutations affecting the quality or quantity of factor VIII (FVIII) manifest as hemophilia A (HA), the most severe congenital bleeding disorder affecting 1 in 5000 males [1]. The severity of the disease is inversely correlated with the residual activity of FVIII, ranging from mild (5–30% activity) to moderate (2–5%) to severe (<1%). When left untreated, individuals with HA suffer spontaneous and prolonged bleeding episodes, leading to increased mortality and morbidity. Modern advancements in medicine have rendered this pathology manageable via intravenous infusions of FVIII from either plasma-derived (pdFVIII) or recombinant (rhFVIII) sources. Treatment is administered either prophylactically or on-demand, and typically begins when a child becomes more mobile (8–12 months), triggering symptoms such as bruising and joint bleeds [2].

The most severe complication in HA treatment is the development of neutralizing antibodies against FVIII, termed inhibitors, in 25–30% of patients with severe HA [3]. Inhibitors typically develop after 15–20 exposure days to FVIII, or at 15–20 months of age, when their immune system is not fully developed and render treatment virtually inefficacious [4–6]. Interestingly, FVIII inhibitors are also prevalent in patients with mild or moderate HA with an incidence of 6.7% after 50 exposures days, reaching up to 13.3% at 100 exposure days [7]. Repeated exposure to FVIII, in a regimen known as immune tolerance induction, is the only proven method to eradicate inhibitors, however it is expensive and is not always successful. Ultimately, the increase in morbidity and healthcare costs associated with inhibitors underlines the urgency to identify strategies that would prevent them from developing at all.

The mechanisms underlying the variability of inhibitor development is unclear, and the ability to reliably predict whether a patient will develop an inhibitor does not currently exist [8]. However, there are many factors that have been associated with increasing this risk. A simplified method of conceptualizing risks for inhibitor development is a binary classification of patient-race, family history [9], FVIII mutation, polymorphisms in MHC-II [10], IL-10 [11], TNF [12], CTLA-4 [13]) and treatment-associated variables (frequency of exposure, type of FVIII concentrate, concurrent infection/inflammation) [14]. In reality however, it is likely a delicate and complex interaction of both sets of variables that influences this immune response.

The decrease in FVIII-inhibitory activity in HIV-positive HA patients has taught us that, mechanistically, the FVIII immune response likely involves some aspects of a classical CD4+ T cell-dependent response in which FVIII is internalized and subsequently degraded by professional APCs, followed by the presentation of FVIII peptides on major-histocompatibility molecule (MHC) class II to a corresponding peptide-specific CD4+ T cell [15,16]. The process of FVIII immunity is complicated by its association with von Willebrand factor (VWF) in the circulation.
This interaction exerts a dominant influence on the biodistribution of the protein characterized by a strong correlation between endogenous VWF levels and infused FVIII half-life, potentially mediated by the masking of FVIII regions to protect it from premature proteolysis by activated protein C, and likely modulating receptor-mediated endocytosis [17–19]. Therefore, a further distinction must be made regarding the continuum between the clearance and antigen presentation of these plasma proteins. In this review, we will examine this paradigm, as well as assess pivotal early steps leading to FVIII immunity at three levels: protein, cellular, and the microenvironment (Fig. 1).

2. Protein characteristics

As proteins circulate in the bloodstream, tertiary structure and post-translational modifications provide binding motifs with varying affinities for different receptors, some of which will mediate proteolysis and clearance, while others will influence presentation. FVIII and VWF are heavily glycosylated proteins with evidence that suggests that both N- and O-linked glycosylations influence their secretion and clearance [20,21]. The roles of these glycan structures have been previously reviewed [22]. Glycomic analysis of the 12 N-linked and 10 O-linked predicted glycosylation sites in plasma derived VWF revealed an estimated 300 unique N-glycan structures and 18 unique O-glycan structures, underlining the variability and the complexity in understanding the roles of these sugars [23,24]. The glycome of FVIII has not yet been investigated in similar detail. FVIII contains a total of 25 potential N-linked glycosylation sites, of which only 20 have been shown to be occupied (Fig. 2) [25,26]. While the sites of potential O-linked glycosylation have not been elucidated, there are at least 7 O-linked glycans exclusive to the B domain that are capped with sialic acid residues [22,27]. The glycans that are exposed when FVIII is in complex with VWF remain unclear. Interestingly, studies using B-domain deleted rhFVIII have not reported differences in clearance or immunogenicity, despite the deletion of a substantial segment of highly glycosylated protein [4,28]. These findings suggest that the glycans outside the B domain may play a dominant role in regulating clearance and immunogenicity.

Mass spectrometry analysis of the sugar chains on pdFVIII and rhFVIII have revealed the presence of high mannose- and complex-type residues on both proteins, with the two sources of FVIII having similar half-lives in primate models [29]. The two high mannose glycan structures remain conserved among recombinant and native proteins, and are located in the A1 and C1 domains (Asn239, Asn2118, respectively) [26,29,30]. The glycan structures on these proteins likely contribute to the stability and folding of FVIII and its pairing with VWF. In particular, mannose glycans on FVIII in the B domain, contribute to ER trafficking and protein folding via binding to LMAN1/ERGIC [20,31]. Mutations in LMAN1 have been linked to combined factor V and FVIII deficiency [32]. In contrast, exposed glycans have also been implicated in clearance and immunogenicity and will be discussed in subsequent sections of this review.

Fig. 1. Three levels of potential modulators of FVIII innate immunity. The FVIII/VWF complex contains characteristics at the protein level that influence endocytosis such as the surface glycosylations, and elements of the 3-dimensional protein itself, which are additionally influenced by shear forces on VWF. FVIII innate immunity is dictated by the receptor and the corresponding cell type that leads to protein degradation, or presentation of peptides on MHC class II. The subsequent development of an adaptive immune response is ultimately dependent on the stable expression of MHC class II and the up-regulation of co-stimulatory molecules CD80 and CD86. These signals are influenced by the state of the surrounding microenvironment, such that danger signals promote the development of immunity, while anti-inflammatory cytokines or the absence of cytokines promote tolerance.

Fig. 2. Post-translational modifications of circulating FVIII and known receptor binding sites. FVIII is highly glycosylated, containing at least 20 N-linked glycans (-N for N-linked, -M for high-mannose) and 7 O-linked (-O) glycan, the latter located exclusively in the B domain. Some of these glycans function as ligands for lectin and/or scavenger receptors and are depicted in their respective domains. The lectin receptors CD206 and CLEC4M are specific for the conserved high-mannose glycans located at Asn239 and Asn2118. The ASGPR binds to desialylated glycans located in the B domain, and LRPI binds to an undefined motif in the light chain. Receptors in red are inhibited for FVIII binding in the presence of VWF.
Major differences between the FVIII products used as replacement therapies in HA are related to glycosylation (Table 1). One obvious difference is the presence of ABO blood group determinants on pdFVIII that do not exist in rhFVIII produced in mammalian cell cultures [26]. Furthermore, humans exhibit inactivations of the cytidine monophosphate N-acetylneuraminic acid gene (CMAH) and the x1,3GT gene, resulting in the inability to convert N-acetyl neuraminic acid (Neu5Ac) to N-glycoly neuraminic acid (Neu5Gc), and an inability to express Gal[x1-3]Gal, respectively [33,34]. As a result, all currently marketed rhFVIII products express Neu5Gc whereas native human FVIII does not. Interestingly, healthy individuals have greatly varying levels of circulating immunoglobulin M (IgM) and immunoglobulin G (IgG) specific against Neu5Gc and Gal[x1-3]Gal [34,35]. These antibodies may influence the immunogenicity and half-life of infused rhFVIII. Anti-gal antibodies have indeed been exploited as mechanisms of organogenesis and HIV vaccines in APCs, and have been implicated in xenograft rejection via complement-mediated cytosis [35]. Although speculative, it has been suggested that dietary Neu5Gc, predominantly in red meat, is associated with the persistent circulation of the antibody-glycan complexes leading to chronic inflammation, a theory that may have implications for prophylactic rhFVIII therapy [36]. Along these lines, a single study in mice has shown that the removal of N-linked glycans is associated with a 30% decrease in FVIII activity and a minor decrease in subcutaneous immunogenicity [37]. Furthermore, studies with inactive FVIII mutants have suggested that the immune response against FVIII is independent of its procoagulant function, but rather involves intrinsic elements of the protein structure [38]. The role of glycosylation in FVIII immunity therefore warrants recognition as it likely influences the localization of FVIII to various scavenger and lectin receptors.

### 3. Biodistribution of VWF and FVIII

Two predominant themes represent the current view of FVIII and VWF biodistribution: that clearance and immunity occur in the liver and spleen respectively, and that VWF is the dominant influence on FVIII distribution. The anatomic distribution of intravenously infused proteins is a multivariate process dependent on the binding affinity to different receptors and also by the formation of immunoglobulin- and complement-mediated immune complexes. Localization of coagulation factors has been primarily assessed in murine models of HA or von Willebrand’s disease (VWD). Upon intravenous infusion, it is likely that the majority of FVIII complexes immediately with VWF, such that only approximately 2–5% of FVIII remains unbound [39,40]. It has been shown that the relative uptake of FVIII and VWF by tissue mass is greatest in the spleen followed by the liver, suggesting a higher affinity of receptor-mediated clearance in the spleen [41,42]. However the sheer size difference between the two organs suggests that the bulk uptake of the two factors is mediated primarily by the liver. As little as 5 min after intravenous infusion, immunofluorescent staining has revealed that VWF and FVIII primarily associate with Kupffer cells in the liver and potentially marginal zone metallophilic macrophages in the spleen, respectively [41,42]. The subsequent depletion of macrophages using clodronate-containing liposomes and gadolinium chloride resulted in increased FVIII and VWF half-life, as well as a decreased FVIII immune response [41,42]. Whether the fate of unbound FVIII parallels that of the FVIII/VWF complex remains unclear. Indeed, FVIII infusion in the absence of VWF dramatically decreases its association with the splenic marginal zone, possibly suggesting increased proteolysis, or perhaps an entirely different location and mode of clearance [43]. However, given the supraphysiologic doses of FVIII infused in order to facilitate detection, these studies should be interpreted with caution.

Investigations of FVIII immunogenicity in animal models have mainly focused on the spleen, with the rationale that high hepatic levels of interleukin (IL)-10 and transforming growth factor-β (TGF-β) in the liver promote tolerance and FVIII/VWF clearance [44,45]. However, increasing evidence suggests that the liver functions as a secondary lymphoid organ, such that liver sinusoidal endothelial cells (LSECs), hepatic stellate cells, and Kupffer cells express MHC class II and co-stimulatory molecules to potentially act as APCs to stimulate CD4+ and CD8+ T cells [46–49]. More insight is needed to establish the balance between hepatic tolerance and immunity. The role of extra-splenic FVIII clearance and presentation may reveal insights into the initiation of FVIII immunity when considering that splenectomized mice still develop inhibitors [41].

Similarly, immune cell interactions with FVIII and VWF have predominantly focused on progenitor-derived APCs, that may not recapitulate important details of the native APC populations involved in processing these proteins. The splenic marginal zone represents an area of particular importance for FVIII innate immunity as it comprises of a wide array of antigen-presenting cells (APCs), optimally located as a divisive line between blood-borne antigens and the adaptive immune cells of the white pulp. The resident APCs: DCs, marginal zone (MZ) macrophages, MZ metallophilic macrophages, and MZ B cells, express a plethora of innate pattern recognition receptors (PRRs) such as toll-like (TLRs) and C-type lectin receptors (CLRs) that facilitate the recognition of a wide array of antigens [50]. These specific primary cell subsets have not been investigated in the context of FVIII and VWF. Recently, it was shown in an in vitro model, that nearly 80% of FVIII associates with the pan B cell marker CD45R B220, which suggests a previously unreported potential role for B cells in the early stages of the FVIII immune response [51].

### 4. FVIII-antigen presenting cell interactions: clearance and presentation?

The FVIII immune response begins with the internalization and subsequent proteolytic degradation by professional APCs, followed by the presentation of FVIII peptides to CD4+ T cells on major-histocompatibility molecule (MHC) class II [15,16]. An additional layer of complexity involves the influence of VWF on the potential masking of FVIII internalization motifs, as well as the apparent dominant role of VWF on FVIII clearance. The modulatory role of VWF on FVIII immunity remains controversial and inconsistent in
| Family | Receptor | Receptor specificity | Expression | FVIII binding | VWF binding | Reference |
|-------|----------|----------------------|------------|----------------|-------------|-----------|
| Lectin | Asialoglycoprotein | Non-reducing terminal b-D-Gal or GalNAc | Hepatocytes | Desialylated B-domain associated glycans, Desialylated N-linked glycans | [79] |
|       | Siglec-5 | Sialic acid | Monocytes, macrophages, neutrophils, B cells | Sialic acid independent | Sialic acid on N-linked glycans | [72] |
|       | CLEC4M (L-Sign) | High mannose glycans | Liver, lymphatic sinusoidal endothelial cells | High mannose glycans | N-linked glycans | [83,84] |
|       | CD206 | Terminal mannose, GlcNAc, fucose | Macrophages, dendritic cells | Exposed mannosylated glycans (inhibited by VWF) | [82] |
| LDL receptor family | LDL receptor (LDLR) | Low density lipoproteins, Ubiquitous | Yes (specific site unknown) | Unknown | [68] |
|       | LRP1 | RAP, PDGF, FVIIa, tPA, fibronectin, A2/A3 domain (when thrombin activated), -VLDL, etc | Hepatocytes, Kupffer cells, DCs, macrophages, neurons, vascular smooth muscle | Under conditions of shear, light chain (blocked by VWF), shear | [61–64] |
| Scavenger | Scavenger receptor (SR) | Hyaluronin, phosphatidylserine | Liver sinusoidal endothelial cells | VWF-dependent FVIII endocytosis | [110] |
|       | SCARA5 | Bacteria (lipopolysaccharide), L-Ferritin | Interstitial fibroblasts | Calcium dependent | [111] |

The substantial post-translational additions of carbohydrate moieties on FVIII and VWF influence their ability to be internalized through recognition by lectin receptors. The impact of glycosylation on the circulating level of plasma proteins has been demonstrated in individuals with different ABO(H) blood group antigens, where blood type O is associated with a 25–30% lower VWF antigen level, and by extension FVIII antigen, compared to non-O blood types due to a decreased half-life of VWF lacking in type A and B glycans [71]. However, the specific endocytic receptors responsible for this discrepancy have not been established.
The glycans on FVIII and VWF are heavily sialylated chain-terminating residues that have implications on uptake. The sialic acid-binding immunoglobulin-type lectin (Siglec) family, specifically Siglec-5, has been implicated to include potential receptors for FVIII and VWF [72]. Neuraminidase treatment abolished binding to VWF but not FVIII, which suggests an alternative binding site for FVIII. Receptors in the Siglec family have evolved as paired isoforms with nearly identical ligand-binding domains and yet elicit divergent immune modulating functions [73,74]. In particular, Siglec-5 has been shown to attenuate innate immune responses in phagocytes, whereas its counterpart, Siglec-14, counteracts this suppression by production of pro-inflammatory cytokines in response to the same pathogen [75]. Similarly, co-ligation of the B cell receptor together with Siglec-2 (CD22) using FVIII-liposomes induces B cell apoptosis leading to peripheral B cell tolerance, which supports the concept that Siglecs represent a mechanism to ensure self-tolerance [76]. This complexity, coupled with the substantial tissue distribution of Siglecs and their isoforms, suggests differential roles in clearance and immunity, an example being the expression of Siglec-5 in myeloid and lymphoid immune cells in the spleen and lymph nodes, but also in the liver [77,78].

In contrast, it has been demonstrated that the asialoglycoprotein receptor (ASGPR), a C-type lectin expressed abundantly in the liver that selectively binds desialylated glycans (non-reducing terminal β-α-galactose or NN-acetyl-α-galactosamine), complexes, primarily with the B domain of FVIII [79]. Hyposialylated VWF has also exhibited a decreased circulating half-life through a ASGPR-dependent mechanism [80]. While ASGPR has not been addressed with an immunological perspective, there are a wide array of endocytic C-type lectin receptors (e.g. CLEC family, DEC205, etc) expressed on DCs that have been shown to induce potent T helper cell responses, but have not been investigated with respect to FVIII and VWF [81].

Two of the N-linked structures at Asn239 and Asn2118, on A1 and C1 domains of FVIII respectively, are conserved high-mannose residues and have been previously shown to facilitate uptake by the macrophage mannose receptor (CD206) on human DCs [82]. However, conflicting evidence has shown that FVIII uptake appears to be largely unaffected by saturating and blocking CD206, which suggests that while this receptor can bind FVIII, it is not an obligate receptor, and that there are compensatory mechanisms to ensure FVIII uptake [66]. Recently, a novel C-type lectin receptor, CLEC4M (also known as CD299, L-SIGN, or DC-SIGNR), was characterized as a novel receptor that binds, internalizes and clears both VWF and FVIII [83,84]. The binding to CLEC4M, expressed on sinusoidal endothelial cells of the liver and lymphatic system, is dependent on exposed high mannose glycans on FVIII, however their influence was not observed in mouse clearance studies [85]. This interaction may have a role in FVIII immunogenicity, as CLEC4M has been shown to play a protective role against coronavirus infection leading to decreased susceptibility and increased viral degradation [86].

4.3. Endosomal sorting

In order to elicit immunity or peripheral tolerance, exogenous antigens must be degraded and presented on MHC class II. However, upon endocytosis, the molecular basis of endosomal sorting that determines antigen presentation versus antigen recycling is only partially understood. Furthermore, while there is a significant body of literature regarding MHC class II presentation in DCs, the capabilities and regulation of other less conventional APCs remain unclear. In the classical sense of antigen presentation, endosomes in DCs fuse with MHC class II-bearing endosomes to load FVIII-derived peptides. Stable expression of peptide-bearing MHC class II molecules on the surface of the cell is primarily governed by the maturation status of the DC. Immature DCs ubiquitinate the MHC class II-peptide complex within the endosomal vesicles and the protein complex is subsequently delivered to the lysosome for degradation. In maturing DCs, the ubiquitination is halted, thus inhibiting the sorting of MHC class II-bearing endosomes to the lysosome, and as a result, the complex is delivered and stably expressed at the plasma membrane [87]. The emerging role of ubiquitination as a regulator of antigen presentation will have significant impacts on immunotherapies targeting specific cell types as well as controlling the reactivity states of DCs leading to either immunity or tolerance. Ultimately, this regulation is dependent on APC maturation, which in turn, is governed by exposure to stimuli in the surrounding microenvironment.

5. The microenvironment: danger theory

A long-standing pillar of immunology focuses on the idea that the immune system distinguishes between self and non-self. However, this model fails to justify a number of findings, such as anergy against “self changes” (i.e. tumours, ageing, pregnancy), and vaccine inefficacy without the inclusion of adjuvants. Matzinger’s danger model presents an alternative paradigm in which, rather than differentiating self from non-self, the immune response is initiated by “danger signals” represented by danger-(DAMPs) and pathogen-associated (PAMPs) molecular patterns [88]. These signals can bind PRRs such as TLRs, NOD-like receptors (NLRs), CLR, and RIG-I-like receptors (RLRs), that rapidly trigger the production of pro-inflammatory cytokines [89,90]. Detection by PRRs also provides a pivotal bridge connecting to the adaptive immune response through the activation/maturation of APCs and subsequent up-regulation of co-stimulatory molecules, both of which are essential components in their interaction with T cells [91]. This mechanism highlights the current model of the FVIII innate response, where robust immune responses are elicited by mature APCs, and the induction of tolerance is mediated by immature APCs [92–94].

An in vitro study has suggested that rhFVIII either alone, or in complex with VWF, does not present danger signals to human monocyte-derived DCs, which suggests that additional external influences, likely associated with the APC microenvironment, may be needed to promote the immune response [95]. Indeed, a survey conducted across 42 HA treatment centres showed a significant concern that administering FVIII replacement therapy in the presence of inflammatory stimuli, particularly surgery, would increase the risk of inhibitor development [8]. Surgical procedures have indeed been associated with a near 3-fold increase in inhibitor risk patients, however these observations could not be confirmed in a murine model without direct application of inflammatory ligands [6,96]. Vaccination has also been hypothesized as a risk factor for inhibitor development, however this theory has been challenged by clinical evidence citing a lack of association between vaccination and inhibitor development in previously untreated patients [97]. Moreover, it has been shown that vaccination in a humanized murine HA model in fact decreases the incidence of inhibitors through a mechanism of antigenic competition involving chemotactic immune diversion [98].

6. The APC-T cell boundary

CD4+ T helper (Th) cells are fundamental to the development of robust adaptive immune responses and the maintenance of self-tolerance. This breadth of function is predominantly attributed to the variety of effector subsets into which Th cells can differentiate upon physical interactions with peptide-bearing MHC class II (signal 1) and co-stimulatory molecules (signal 2) expressed on
antigen presenting cells (APC). The polarization of naïve T cells and subsequent proliferation is further influenced by the magnitude of co-stimulation as well as by cytokines in the surrounding microenvironment (signal 3).

Healthy individuals and HA patients, with and without inhibitors, have been shown to have interferon-gamma (IFN-γ) secreting CD4+ T cells, indicating a basal, and potentially initiating, role of Th1 cells on FVIII immunity [99]. Furthermore, a study of T-cell clones isolated from brothers with mild HA discordant for inhibitor status revealed that a Th1/Th1 response dominates early in the immune process before transitioning into Th2-polarized cells [100]. In parallel with Matzinger’s danger theory, these findings suggest an important role for a pro-inflammatory response in the propagation of FVIII inhibitors.

Interestingly, the overlay of HMC-presented peptides and the corresponding T- and B-cell epitopes are primarily situated in the A2 and C2 domains as previously reviewed [15,101,102]. These findings may suggest that MHC polymorphisms and T and B cell clones are more frequently directed to those domains in a somewhat evolutionarily conserved manner. Coincidentally, it has been recently hypothesized that the development of self-reactive ADAMTS13 antibodies in acquired thrombotic thrombocytopenic purpura is associated with molecular mimicry of ADAMTS13- and pathogen-derived MHC class II presented peptides [103]. Indeed, the presence of potentially cross-reactive low affinity FVIII-reactive antibodies in the circulation of healthy patients may suggest an inherent pathogen-mediated natural immunity against the protein that may influence clearance and adaptive immune responses [58,104].

7. Tolerance at the innate level

Few techniques have been employed in an attempt to reduce and prevent inhibitors before they manifest. One obvious strategy is to limit the presence of pro-inflammatory signals, thereby inhibiting APC maturation. Recently it was shown in two HA mouse models that transient co-administration of FVIII with the steroidal, dexamethasone, significantly decreases the titre and incidence of FVIII antibodies and inhibitors, and results in a durable thymic regulatory T cell (Treg)-mediated tolerance [105–107]. Additionally, it was reported for two murine strains of HA that administration of a depleting anti-CD3 antibody increases the percentage of Treg cells in the spleen and significantly decreases the production of FVIII inhibitors [108]. Unfortunately, these methods of immunomodulation are likely to be accompanied by an albeit small increase in the risk of opportunistic infections in young HA patients. Another immunomodulatory approach that has recently shown promise in murine models is the initial immune priming via oral delivery of plant cells expressing FVIII antigens to target the tolerogenic mucosal environment. Following intravenous infusion of full-length FVIII, a substantial suppression of anti-FVIII responses was observed that was attributed to an upregulation of immunosuppressive cytokines typical of the liver microenvironment (IL-10 and TGF-β), and the induction of FVIII-specific Tregs [109].

8. Conclusions

A significant proportion of research has been dedicated to assessing the adaptive immune responses and reversion back to tolerance after the immune response to FVIII has already developed. However, there is a need for developing a tolerogenic method of administering FVIII as well as identifying potential biomarkers or predictive assays to determine inhibitor risks in children before they begin FVIII replacement therapy. The immune response may initiate as soon as FVIII enters the circulation, highlighting the critical role of the innate immune system in governing the balance between clearance and immunogenicity. Future studies on the prevention of inhibitors need to address the multifactorial complexity of the FVIII innate response: the microenvironment, the cells, and the inherent characteristics of the FVIII protein.

Author contributions

Contributions: J.L. wrote the first draft of the manuscript. M.G., C.H., and D.L. edited the manuscript.

Conflict of interest disclosure

The authors declare no competing financial interests.

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