



Research paper

War and peace: Factor VIII and the adaptive immune response

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ABSTRACT

The development of neutralizing anti-factor VIII (FVIII) antibodies (inhibitors) remains a major challenge for FVIII replacement therapy in hemophilia A patients. The adaptive immune response plays a crucial role in the development and maintenance of inhibitors. In this review, we focus on our current understanding of FVIII interactions with cells of the adaptive immune system and the phenotype of the resultant response. Additionally, we examine both current and novel FVIII tolerance induction methods that function at the level of the adaptive immune response.

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1. Introduction

Hemophilia A is an X-linked recessive bleeding disorder characterized by quantitative or qualitative defects in factor VIII (FVIII), an essential protein co-factor of the coagulation cascade. The condition is characterized by prolonged provoked bleeding, and in severe cases (<1% normal plasma FVIII levels) spontaneous bleeding into joints and soft tissues. Bleeding symptoms are ameliorated via replacement therapy with either recombinant or plasma-derived FVIII concentrates. The major complication associated with this treatment is the development of neutralizing anti-FVIII antibodies (inhibitors), a phenomenon that occurs in approximately 30% of treated severe hemophilia A patients. By binding to functionally essential regions of FVIII, inhibitors render subsequent FVIII replacement therapy ineffective, leaving patients vulnerable to bleeding symptoms and increasing the risk of morbidity.

By understanding the FVIII immune response one can potentially identify cellular targets to promote tolerance or, in cases where the anti-FVIII immune response has already been established, restore tolerance to the protein. Early events of the FVIII immune response and the protein's interactions with the innate immune system are discussed in another review in this issue. Here, we focus on interactions of FVIII with the adaptive immune system, and its role in the establishment and maintenance of inhibitors. We also introduce current and future FVIII tolerance

induction methods targeted at the level of the adaptive immune response (Table 1).

2. FVIII and the cells of the adaptive immune system

2.1. The Primary Immune Response to FVIII

The primary immune response (Fig. 1A) is initiated by antigen uptake via antigen presenting cells (APCs) and subsequent antigen presentation to cognate CD4⁺ T cells in the presence of an activating, pro-inflammatory microenvironment [1]. This process is reviewed in more detail elsewhere in this issue. In brief, activation of a CD4⁺ T cell to an effector T cell (Teff) is dependent on antigen presentation by the APC (Signal 1), upregulation of co-stimulatory molecules CD40 and CD80/86 by the APC (Signal 2) and pro-inflammatory cytokine production (Signal 3). Following activation, Teffs migrate from the periaarterial sheath to the B cell follicles of the spleen. Naïve B cells that have FVIII-specific B cell receptors (BCRs) will have endocytosed and processed the antigen to present its peptides via major histocompatibility complex (MHC) II. FVIII-presenting B cells will then interact with the TCR of the activated T cell and engage co-stimulatory molecules (CD40L and CD28 on the T cell with CD40 and CD80/86 on the B cell).

In hemophiliacs lacking FVIII production, mechanisms of central tolerance leading to deletion of T and B cell clones with reactivity to FVIII will be absent. Interestingly, these mechanisms appear to be innately incomplete, as there has been evidence of FVIII-reactive T cells and anti-FVIII antibodies even in normal individuals [2,3].

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Table 1

Novel methods for FVIII tolerance induction targeted to the adaptive immune response.

	Therapy	Mechanism of action
Anti-T Cell Strategies	Anti-CD3	Prevents formation of activating APC-T cell synapse and increases Treg:Teff ratio [42]
	Rapamycin	Increases Treg:Teff ratio [43]
Anti-B Cell Strategies	Engineered FVIII-specific Tregs	Inhibition of Teff proliferation by antigen-specific Tregs [44]
	Antigenic liposomes with CD22 ligand	B cell suppression via engagement of CD22 [45]
	B cell blasts expressing A2 and C2 IgG fusion proteins	Treg recruitment [46]
Anti-plasma Cell Strategies	Bortezomib	Non-specific eradication of plasma cells [57]

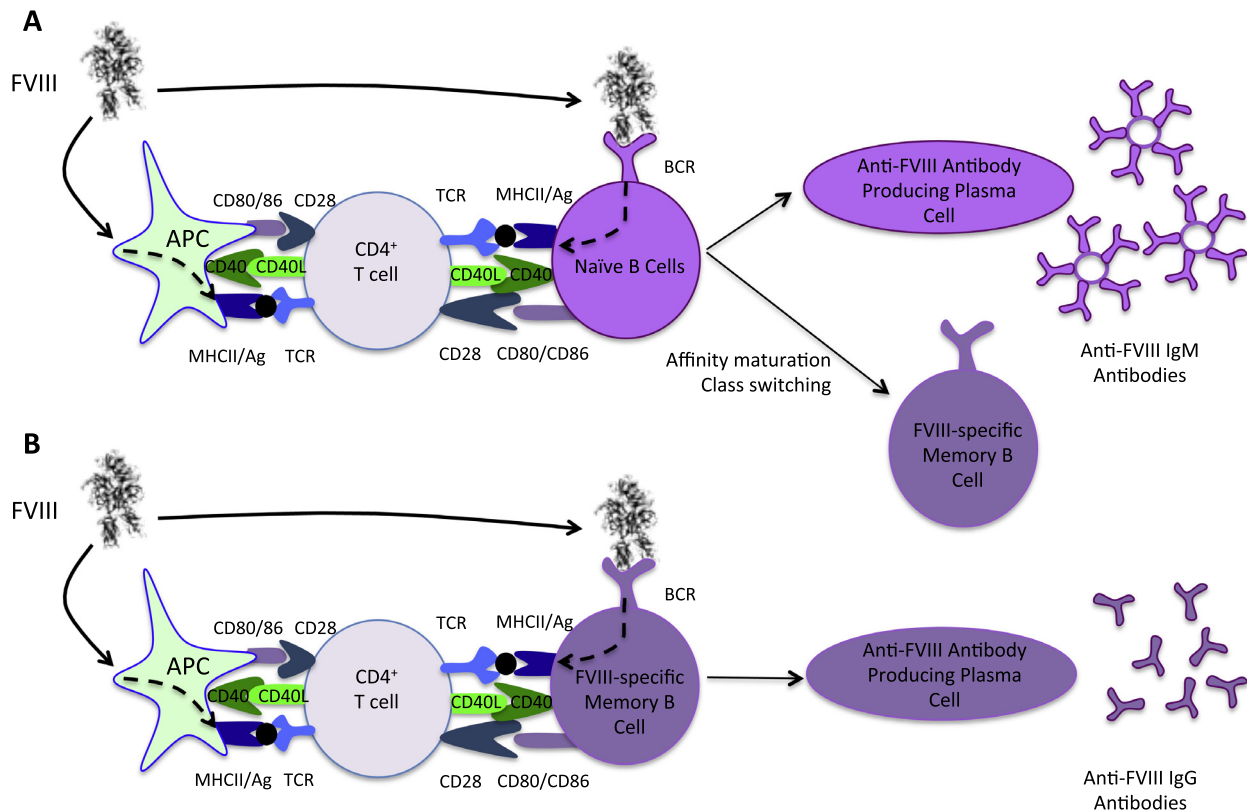


Fig. 1. A. The primary immune response to FVIII. Upon primary exposure to FVIII, APCs will endocytose the antigen and present its peptides to cognate CD4⁺ T cells. In the presence of an activating, pro-inflammatory microenvironment (upregulation of co-stimulatory molecules CD40 and CD80/86 by the APC, production of pro-inflammatory cytokines) this results in activation of the T cell to Teff. Naïve B cells that have FVIII-specific BCRs will have also endocytosed and processed FVIII to present its peptides via MHCII. FVIII-presenting B cells will then interact with the TCR of the activated T cell and engage co-stimulatory molecules (CD40L and CD28 on the T cell with CD40 and CD80/86 on the B cell). This interaction results in B cell activation, proliferation and differentiation. A proportion of these B cells will terminally differentiate into anti-FVIII IgM antibody producing plasma cells. Others will undergo affinity maturation and class-switching to form FVIII-specific memory B cells. B. The secondary immune response to FVIII. Upon secondary exposure to FVIII, similar events to the ones in the primary immune response will be observed.

It can therefore be expected that hemophilia A patients will have some circulating cognate T and B cells capable of forming B-T cell synapses. This interaction results in B cell activation, proliferation and differentiation. A proportion of these B cells will terminally differentiate into anti-FVIII antibody producing plasma cells, while others will undergo affinity maturation and class-switching to form FVIII-specific memory B cells. These cells will play a key role in mounting a robust immune response upon subsequent re-exposure to FVIII.

2.2. The secondary immune response to FVIII

During the primary immune response, the T and B cells responsible for mounting the anti-FVIII immune response are naïve. In contrast, the secondary immune response (Fig. 1B) involves

FVIII-specific memory B cells. These cells are able to mount a response to FVIII much quicker than naïve B cells, and their involvement results in the production of higher affinity neutralizing antibodies to FVIII. Studies suggest that the frequency of peripheral FVIII-specific memory B cells in hemophilia A patients with inhibitors range from <0.01 to 0.40% of total IgG positive B cells [4]. The frequency of FVIII-specific B cells appeared to be affected by the presence of recent antigenic challenge, but did not correlate with anti-FVIII antibody titers [4].

2.3. Effector T cells versus regulatory T Cells

As described, Teffs play a crucial role in the initiation and maintenance of an effective anti-FVIII immune response. The importance of this cell type was highlighted by observations derived

from hemophilia A patients who were positive for both inhibitors and human immunodeficiency virus (HIV) infection. As the HIV infection progressed in these individuals, the associated decrease in their CD4⁺ T cell population correlated with a decrease in FVIII inhibitory activity [5]. Since then, further evidence has emerged to confirm the role of CD4⁺ T cells in the anti-FVIII immune response [6].

Regulatory T cells (Tregs) also play an important role in controlling the FVIII immune response. These cells are characterized by expression of CD4, CD25 and FoxP3 [7,8]. In contrast to T cells, Tregs suppress immune responses through either direct cell-to-cell contact or through the production of anti-inflammatory cytokines such as IL-10 or TGF- β [9]. Natural Tregs can be derived in the thymus during the central T cell selection process, or they can develop from appropriately stimulated CD4⁺ T cells in the periphery [10,11]. A number of studies have demonstrated that decreased numbers of Tregs are associated with autoimmune disease, inflammatory bowel disease, and graft rejection, suggesting a crucial role for Tregs in the maintenance of tolerance to both self and non-self antigens [12]. The balance between T cells and Tregs is therefore very likely to be a key contributor to the decision of whether exposure to FVIII in hemophilia A patients results in an immunogenic or tolerogenic response.

3. Phenotype of the anti-FVIII immune response

Longitudinal studies looking at the emergence of the primary anti-FVIII immune response in hemophilia A patients are currently lacking. Our understanding of this process therefore relies on data from murine studies (described below) and our general understanding of how the human body mounts an immune response. It is therefore thought that in humans, the primary anti-FVIII immune response predominantly consists of weaker binding IgM antibodies. The progression to a secondary immune response is marked by emergence of IgG antibodies. While all four IgG subclasses have been associated with the presence of inhibitors in hemophilia A patients, IgG1 and IgG4 have been found to be more prominent than IgG2 and IgG3 [13,14]. While IgG1 is present in both low-titer (<5 BU/ml) and high-titer (>5 BU/ml) inhibitor patients, IgG4 is found at high concentrations in high-titer patients [13]. Recent studies have also indicated that the emergence of high affinity IgG anti-FVIII antibodies predominantly of IgG4 subclass (demonstrated in a competition ELISA protocol), is an early indicator of the emergence of inhibitory anti-FVIII antibodies [15].

Although the least abundant IgG subclass, IgG4 antibodies possess a number of unique properties. Foremost, they have been shown to engage in Fab arm exchange with each other, combining specificities of two IgG4 molecules and resulting in monovalent bispecific antibodies [16]. They are therefore unable to cause cross-linking. In addition IgG4 cannot activate complement or Fc-receptors and are therefore associated with non-inflammatory immune responses. This antibody subclass is associated with long-term exposure to antigens in non-infectious settings (e.g. long-term bee keepers and those undergoing immune therapy for allergies) [17]. Finally, IgG4 has been associated with antibody responses against adalimumab, a therapeutic monoclonal antibody against TNF- α [18].

The anti-FVIII immune response is polyclonal [19], however the A2 and C2 domains have been found to be the immunodominant regions of the protein [20]. The A2 domain of FVIII is responsible for the protein's interaction with activated FIX (FIXa), while the C2 domain interacts with phospholipid surfaces. Inhibitors targeted to these functional regions would therefore significantly impair the procoagulant activity of FVIII, by limiting its ability to bring FIXa and FX together at the phospholipid membrane.

A number of genetic factors have been associated with an increased risk of inhibitor development in hemophilia A patients. These include ethnicity, severity of FVIII mutation, polymorphisms in the MHC II [21] genes as well as polymorphisms resulting in increased expression of TNF- α and IL-10 [22,23]. However, these non-modifiable factors do not fully predict a patient's risk of developing an inhibitor. Treatment intensity and regimen (prophylaxis or on-demand), age of first exposure to FVIII, and even the type of FVIII concentrate may also contribute to a patient's likelihood of developing an inhibitor [24,25].

Currently, the majority of novel FVIII tolerance induction methods are initially studied in murine models of hemophilia A. It is therefore important to identify similarities and differences between the human and murine anti-FVIII immune response. Similar to humans, the primary anti-FVIII immune response in mice consists of IgM antibodies [26]. The secondary immune response is characterized by the appearance of IgG1 (more prominent) and IgG2 antibodies. The magnitude of the anti-FVIII immune response in mice has been shown to be affected by the specific inbred mouse strain. While IgG1 titers were similar between Balb/c and C57Bl6 hemophilic mice, IgG2 titers were twice as high in the latter [26]. The most noteworthy difference between the human and murine anti-FVIII immune response is the absence of IgG4 subclass antibodies in mice [27]. This represents a significant challenge when assessing the potential for translating FVIII tolerance induction methods that are successful in mouse models into clinical practice. This is especially problematic for tolerance induction therapies that suppress the murine anti-FVIII immune response through the generation of Tregs. Some peripherally derived Treg subsets exert their suppressive effects through the production of IL-10, a cytokine with pleiotropic influences. As previously mentioned, increases in this cytokine have been identified as one of the genetic factors associated with an enhanced risk of inhibitor development in hemophilia A patients. Furthermore, IL-10 is known to drive IgG4 production in humans [28,29]. Therefore, the role of IL-10 as a tolerance inducing cytokine in mouse models of FVIII inhibitors needs to be interpreted with caution in terms of clinical relevance.

4. Current FVIII tolerance induction therapies

Immune tolerance induction (ITI) therapy remains the only therapy used to restore FVIII tolerance in hemophilia A patients with inhibitors. ITI consists of repeated administration of either low- or high-dose FVIII over prolonged periods of time (months to several years) leading to eradication of the inhibitor in 70–85% of patients [30]. Although widely used in the clinic, the mechanisms through which this therapy works remain unknown.

There has been evidence both *in vivo* and *in vitro* to suggest that high-dose FVIII causes apoptosis of murine FVIII-specific memory B cells [31]. However, the lowest FVIII dose required to completely inhibit these FVIII-specific B cells (10 μ g/ml, approximately 100 IU/ml) is higher than can be achieved with even the high dose ITI protocols and does not account for the efficacy of low-dose ITI protocols. Although the B cell apoptotic process was shown to be dependent on Fas and caspase activation [31], the mechanistic details of this outcome requires further elucidation. In humans, ITI success has been correlated with the eradication of anti-FVIII IgG4 [13]. There has also been limited evidence to suggest a role for anti-idiotypic antibodies in the success of ITI in inhibitor population [32]. This observation was corroborated by the ability of anti-idiotypic antibodies against a monoclonal inhibitor of the FVIII C2 domain to restore normal FVIII activity in a murine hemophilia A model [33]. It is possible that these anti-idiotypic antibodies not only neutralize inhibitory anti-FVIII antibodies and the

corresponding FVIII-specific B cells, but may also inhibit FVIII-specific B cells by crosslinking the BCR with the inhibitory FcγRIIB receptor on these cells [34].

ITI is only effective in restoring FVIII tolerance in 70–85% of patients with inhibitors [30] and has an associated inhibitor recurrence risk of 30% [35]. The combination of this therapy with rituximab has been shown to increase ITI success rate [36]. Rituximab is a chimeric monoclonal anti-CD20 antibody (human constant regions, murine variable regions) of the IgG1 isotype that causes non-specific apoptosis of B cells at all stages of development [37]. Studies suggest that the effects of rituximab can be attributed to pro-apoptotic signaling, complement dependent cytotoxicity and antibody dependent cellular cytotoxicity of B cells [38]. However, due to the lack of antigen specificity, the use of rituximab results in off-target effects leaving the patient immunocompromised for extended periods of time. In a hemophilia A mouse model, anti-CD20 was also shown to inhibit the anti-FVIII immune response during a high-dose FVIII treatment designed to mimic ITI [39]. This effect was accompanied by an increase in Tregs [39]. Due to ethical and technical challenges of repeated IV administration, ITI protocols performed in murine models are only abbreviated versions of ITI protocols used in hemophilia A patients. In mice, repeated high-dose FVIII administration is associated with an increase in inhibitor titer rather than tolerance induction. The use of hemophilia A mice to determine the mechanism of action for combining ITI with anti-CD20 therefore has significant challenges.

Aside from its limited success rate, ITI is also expensive and inconvenient to administer. In young boys, to facilitate repeat IV infusions, it often requires insertion of central venous catheters, which are associated with a risk of infection, thrombosis and mal-function [40]. As a result, novel therapies for FVIII tolerance induction in hemophilia A patients would be welcome.

5. Novel FVIII tolerance induction therapies targeted to the adaptive immune response

5.1. Anti-T Cell Strategies

As previously outlined, T cells (both Teffs and Tregs) play an important role in initiating or preventing the anti-FVIII immune response. Therefore, there have been a number of efforts aimed at either preventing activation of Teffs or increasing the Treg population. During antigen presentation by APCs, engagement of CD3 on CD4⁺ T cells is required for their activation to Teffs. The immunosuppressive properties of anti-CD3 antibody therapy have therefore been explored in the treatment of autoimmune diseases such as type I diabetes [41]. When a non-Fc receptor binding anti-CD3 antibody was administered to hemophilia A mice prior to FVIII replacement therapy, there was a significant increase in the ratio of splenic Tregs to Teffs [42]. This effect was associated with a decrease in both inhibitor incidence and anti-FVIII antibody titers when compared to controls [42]. Other immunosuppressive therapies such rapamycin have also been used in the context of hemophilia A with similar results [43]. Although encouraging, the durability of the tolerance induced by these therapies has not been established. In a more recent effort to harness the tolerance promoting function of Tregs, FVIII-specific Tregs were engineered *ex vivo* using chimeric antigen receptor technology: a TCR isolated from a hemophilia A inhibitor patient T cell clone was transduced into *ex vivo* expanded Tregs from healthy human donors [44]. *In vitro*, these Tregs were able to suppress the proliferation of Teffs with the same TCR specificity [44]. Furthermore, when incubated with splenocytes from FVIII-immunized hemophilia A mice in the presence of FVIII, the engineered Tregs suppressed the generation

of anti-FVIII antibody producing cells as indicated by ELISPOT assay [44]. Although there is the potential for the *ex vivo* production of engineered Tregs in large quantities, a 1–2 Treg to splenocyte ratio had to be used to completely prevent antibody production in the aforementioned assay. However, it is important to note that in this scenario, Tregs with a single TCR clone were able to suppress a polyclonal anti-FVIII immune response. Further studies must be carried out to determine the mechanistic basis and translational potential of this therapy.

5.2. Anti-B Cell Strategies

Based on the limited data currently available, ITI appears to function predominantly at the level of B cells to restore tolerance to FVIII. A number of novel methods have been used to target FVIII-specific B cells with the hope of replacing or improving ITI. There have been some encouraging results using antigenic liposomes that also display CD22 ligands. CD22 belongs to the SIGLEC (sialic acid binding Ig-like lectin) family of lectins and is expressed on the surface of mature B cells, where it functions to suppress BCR signaling in a proximity-dependent manner. Administration of liposomes with both FVIII and CD22 ligands on their surface to naïve hemophilia A mice were shown to result in lower anti-FVIII IgG titers than administration of FVIII alone [45]. This resulted in significant protection from bleeding when these mice were subsequently reconstituted with FVIII [45]. It remains to be seen if these antigenic liposomes can have similar tolerance inducing effects in primed hemophilia A mice that have a pre-existing anti-FVIII immune response. Other efforts to promote FVIII tolerance include transduction of B cell blasts to express A2 and C2 IgG heavy chain fusion proteins [46]. Treatment of naïve hemophilia A mice with these engineered B cells yielded a dampened response to subsequent FVIII challenge. These cells were able to reduce the anti-FVIII immune response even when administered to mice previously immunized with FVIII. The effect was shown to last as long as 2 months, and likely involves the recruitment of Tregs.

5.3. Anti-plasma Cell Strategies

The failure of ITI even when it is administered in combination with rituximab suggests that, in some patients, eradication of anti-FVIII B cells is not sufficient to restore tolerance to FVIII. Long-lived plasma cells may have a role in the maintenance of the anti-FVIII immune response in some individuals. In hemophilia A mice, it has been shown that once the anti-FVIII immune response is established, anti-FVIII antibody producing plasma cells can persist for up to 22 weeks in the absence of additional FVIII administration [47]. This is much longer than the 1–10 day half-life of regular plasma cells [48], further suggesting a role for long-lived plasma cells that have been shown to survive for up to 6 months in the bone marrow [49]. It is important to note that as a B cell matures into a plasma cell, it loses surface expression of antigen-specific IgG. It is therefore virtually impossible to develop a FVIII-specific tolerance induction therapy targeted at plasma cells. Efforts to target anti-FVIII antibody producing plasma cells have therefore been less extensive than those aimed at FVIII-specific B cells.

Bortezomib is a high affinity inhibitor of the 20S proteasome that is used in the treatment of plasma cell myeloma and mantle cell lymphoma [50]. Although its mechanism of action remains to be fully understood, it is thought that bortezomib prevents degradation of pro-apoptotic factors, resulting in programmed cell death and activation of the terminal unfolded protein response [51,52]. A cell's sensitivity to this inhibitor is proportional to its protein output, and due to their high immunoglobulin production, plasma cells are one of the main cell types targeted by this agent

[53]. Bortezomib has also been effective in the treatment and management of graft versus host disease in humans, as well as in experimental models of lupus erythematosus, psoriasis and multiple sclerosis [54–56]. When bortezomib was co-administered with FVIII in naïve hemophilia A mice as a preventative measure, inhibitor onset was significantly delayed [57]. This was accompanied by a marked decrease in anti-FVIII IgG secreting plasma cells in both the spleen and the bone marrow. In contrast, when bortezomib was co-administered with FVIII in FVIII-primed hemophilia A mice, inhibitor progression was only marginally reduced and the effect on plasma cells was lost. The use of a therapeutic that is already available in the clinic for the treatment of other disorders such as multiple myeloma, to enhance the eradication of inhibitors is an attractive possibility. In this case however, bortezomib may be limited by not only its marginal effect on the anti-FVIII immune response in pre-immunized subjects, but also by its potential for producing thrombocytopenia [58].

6. Conclusions

The development of an anti-FVIII immune response in treated hemophilia A patients remains a major clinical challenge and involves both innate and adaptive immune responses. In this review, we have summarized our current knowledge of the interactions between FVIII and the adaptive immune system, as well as the phenotype of the resulting antibody response. We also discussed current and novel FVIII tolerance induction methods that target the cells of the adaptive immune system. Future studies should focus on further elucidation of ITI mechanisms of action so that its efficiency can be enhanced. Efforts to develop alternative FVIII tolerance induction therapies, some of which are described in more detail elsewhere in this issue, should also be continued. Due to the complexity of the immune response, a combination of therapies that target multiple levels of the immune response may be required for optimal outcomes.

Contributions

MG wrote and edited the manuscript. JL, CH and DL edited the manuscript.

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