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Review of Immune Tolerance Induction in hemophilia A

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Abstract

At first sight the bleeding disorder hemophilia A seems to have little in common with immune disorders, but immunology research intersects with other disciplines including hematology.

Nowadays, the most important complication in the treatment of hemophilia A is the development of neutralizing antibodies (inhibitors) against exogenous administered factor VIII (FVIII), which occurs in approximately 30% of all patients with severe hemophilia A. This antibody response renders FVIII replacement therapy ineffective, thereby increasing the risk for uncontrollable bleeding and morbidity, decreasing quality of life and increasing healthcare costs. The only proven effective therapy to eradicate these inhibitors is immune-based. Using a protocol called 'immune tolerance induction' (ITI), the repeated and frequent administration of FVIII under non-inflammatory conditions downregulates the established antibody response and induces immune tolerance.

There has been progress in research clarifying the mechanisms that mediate tolerance induction using ITI, both from patient studies and from research in cell culture and animal-based models. Peripheral tolerance induction to FVIII involves the apoptosis of antigen-specific B-memory cells, anergy induction in antigen-specific effector T-cells (Teff), induction of regulatory T-cells (Treg) and the formation of anti-idiotypic antibodies. In this review hemophilia A will be used as an example to discuss current concepts of tolerance induction as they are applied in patient care. Where possible, we will extrapolate tolerance findings in hemophilia A to related pathways known to affect auto-immune disorders or allergy.

Keywords: Hemophilia A; inhibitors; anti-FVIII antibodies; immune tolerance induction (ITI); working mechanism.

1. Introduction

Hemophilia A is one of the most common inherited bleeding disorders, affecting 1 in 5000 live born boysworldwide[1]. This X-linked disease is caused by a deficiency of the coagulation protein factor VIII (FVIII). Due to this deficiency patients carry a lifelong risk of spontaneous and life-threatening bleeds and they require frequent intravenous infusions of FVIII in order to prevent or treat these bleeding events. The disease has a long history, in which treatment options improved tremendously, evolving from whole blood transfusion to cryoprecipitate to recombinant FVIII (rFVIII) concentrates, including the recently introduced products with an extended half-life. During this evolution many problems and challenges had to be overcome, of which the prevention of viral transmission, like HIV and HCV, by plasma derived products is one of the most striking examples. Nowadays the remaining and challenging complication in the treatment of is the formation of neutralizing antibodies against FVIII ('inhibitors'). Inhibitor development occurs in approximately 30% of all patients with severe hemophilia A and 5% of all patients with moderate or mild hemophilia A[2,3]. As a consequence of these inhibitors traditional replacement therapy becomes ineffective, making it necessary to switch to alternative hemostatic therapies by using bypassing agents, which are less efficient and more costly. . Thereby inhibitors significantly increase morbidity and negatively influences patients' quality of life[4,5].

Given the significant burden of inhibitors on both patients' health and health-care costs many efforts have been made to prevent or eliminate anti-FVIII antibodies. For so far the only effective therapy to eliminate inhibitors is immune tolerance induction (ITI), which is successful in 60-80% of all cases[6,7]. This therapy was first described by Brackmann and Gormsen and the concept of ITI is that repeated and long term administration of FVIII leads to down-regulation of the immune response[8.] It is a very invasive and costly therapy (around €60.000 per month) and it often takes several years in order to achieve tolerance[9]. Despite the 40 years of experience with ITI there are still many issues unresolved, especially regarding the mechanism of inducing tolerance and the optimum ITI regimen, including the dose and type of FVIII. Regarding the latter, there have been reports about the potentially beneficial role of Von Willebrand

Factor (VWF) and the recently introduced recombinant FVIII-Fc fusion protein (rFVIII-Fc) in the induction of tolerance[10–16].

Since hemophilia A is one of the few clinical examples in which an already established immunological problem can be successfully down-regulated with ITI, this disease provides a valuable model for clarifying the mechanisms of tolerance induction. However, information on ITI is scattered and mostly derived from in-vitro studies, animal models or small retrospective cohorts, which are almost by definition inconclusive. We performed a review to summarize all available information on the working mechanism of ITI.

The review will include a discussion of structural aspects of factor FVIII and the pathophysiology of inhibitor formation, followed by working mechanisms proven effective for ITI in hemophilia A. Finally we will also discuss evidence generated from other auto-immune diseases in which return of immune tolerance is desirable as well.

2. Structural aspects of factor VIII

FVIII is predominantly produced by the sinusoidal endothelial cells in the liver and is released in the circulation as a highly glycosylated heterodimeric glycoprotein consisting of 2332 amino acids, composed of 6 domains: A1, A2 and B form the heavy chain (HC) and A3, C1 and C2 the light chain (LC)[17]. After it is secreted, FVIII is non-covalently bound to VWF, which stabilizes FVIII and concentrates it at the site of action[18] (**figure 1**). This binding occurs between the A3 and C2 domains of FVIII and the D'D3 domain of VWF and under physiologic conditions approximately 94% of the FVIII molecules are bound to VWF[18]. Activated FVIII (FVIIIa) is released from VWF following proteolytic cleavage and release of the B-domain. This FVIIIa is able to act as a cofactor to factor IXa to form the intrinsic Xase complex generating thrombin, thereby playing an essential role in the coagulation cascade. Endogenous FVIII has a half-life of 12-16 hours, after which FVIII is eliminated by the liver and probably also by the spleen[19,20]. Of note, the life-cycle of therapeutically administered FVIII resembles that of endogenous FVIII with the exception that for FVIII-preparations without VWF, the binding with endogenous VWF occurs in the circulation instead of in the liver[21].

3. Pathophysiology of inhibitor formation

3.1 Definition of inhibitors and effect on FVIII

Approximately one third of all patients with severe hemophilia A develops inhibitors, usually within the first 50 exposure days with FVIII[3]. These anti-FVIII antibodies are poly-clonal, high-affinity IgG molecules with an overrepresentation of IgG4[22]. Although most FVIII inhibitors have multiple epitope specificities, antibodies targeting the A2 and/or C2 domains of FVIII are the most frequent[23].

Anti-FVIII antibodies counteract the pro-coagulant function of FVIII in several ways[21]. First of all and most frequently the function of FVIII is neutralized by steric hindrance: binding of inhibitors to functional epitopes of FVIII prevents its interaction with some of the partner molecules, thereby interfering with FVIII function in the coagulation cascade[24]. For example, anti-A2 antibodies can impede the binding and activation of FX.

Moreover, some anti-FVIII antibodies have enzymatic activity and can inactivate FVIII by hydrolysis[25,26]. Finally, also antibodies directed to 'non-functional' epitopes, such as A1 and C1, can limit the function of FVIII by lowering its stability and by the formation of immune complexes which accelerate the *in vivo* clearance of FVIII[27–29]. This type of antibodies is not detected by the Bethesda assay, a functional assay that is considered the golden standard test for measuring inhibitor titers. One may speculate that these antibodies may contribute to the shortened half-life of infused FVIII seen in some patients with an undetectable inhibitor level.

3.2 Risk factors of inhibitor formation: A combination of lack of central tolerance and a pro-inflammatory state

The development of FVIII inhibitors is influenced by both genetic and environmental risk factors, that include the conformation of FVIII protein itself, its interaction with the immune system and the inflammatory and immune condition of the patients at the moment of FVIII administration[3,30,31]. These risk factors are summarized in **table 1**.

Table 1. Risk factors of inhibitor formation in hemophilia A[3,30–35].

Genetic / non-modifiable	**Environment / modifiable**
Established	*Established*
Severity of hemophilia	Number of exposure days
Factor VIII (FVIII) gene mutation	Intensity of treatment at first exposure
Family history of inhibitor	
Proposed:	*Proposed:*
Ethnicity	Type of FVIII concentrate
Polymorphisms in immune-response genes	Current infection or inflammatory state

The genetic or non-modifiable risk factors include the FVIII gene mutation and severity of hemophilia, the family history and possibly ethnicity[32,34,35]. Moreover several polymorphisms in immune response genes such as interleukin 10 (IL-10), tumor necrosis factor (TNF) and cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) are identified as genetic risk factors[33,36].Theoretically but not established yet, human leukocyte antigen (HLA) type could be a risk factor in inhibitor formation as well, as it is one of the factors determining which peptides are presented to naive T helper cells[33,34]. The environmental and potentially modifiable risk factors consist of treatment regimen employed (including prophylactic versus on-demand), intensity of treatment at first exposure, type of concentrate used and danger signals at moment of administration, i.e. inflammation caused by for example major bleeds or surgery[3,31,34,37].

The best predictor for inhibitor development appears to be the FVIII genotype, whereby the risks of inhibitors ranges from > 75% in multi-domain deletions trough 20-30% in the intron 22 inversion mutation to < 10% in missense mutations and small FVIII deletions and insertions[32,38]. The strong relationship between FVIII genotype and inhibitor risk is best explained by the fact that hemophilia A patients lack central tolerance to FVIII[20,39,40]. As consequence to the complete absence of autologous FVIII in hemophilia patients, FVIII-specific T- and B-cells can escape the selection procedure to eliminate self-reactive cells, and have increased propensity to become activated after FVIII administration. However, since healthy subjects and hemophilia patients have both demonstrated CD4+ T-cell responses to FVIII and anti-FVIII antibodies, the development of inhibitors is not consequential to

defects in central tolerance induction[29,41,42]. Instead, peripheral tolerance mechanisms might play an instructive role in pathologic manifestations of FVIII-reactive T- and B-cells. These mechanisms include the presentation of self-antigens by dendritic cells (DCs) in steady state to result in anergy or deletion of autoreactive clones, the suppressive activity of regulatory T-cells (Tregs) and anti-idiotypic antibodies that neutralize potential harmful self-reactive antibodies. When also these tolerance mechanisms fail at the background of not having developed central tolerance to FVIII in congenital hemophilia A, an allo-antibody response to FVIII is generated.

Concerning treatment related factors the evidence is less clear. Especially the role of factor product type, i.e. plasma derived FVIII (pdFVIII) versus rFVIII, remains highly debated, whereby some hypothesize that VWF, present in pdFVIII-products, serves as a chaperone for FVIII and protects against inhibitor formation. Many observational studies, meta-analyses and systematic reviews were performed, with overall non-conclusive results[43–45]. In 2016 the SIPPET study (Survey of Inhibitors in Plasma-Product Exposed Toddlers) was published, a randomized controlled trial with the aim of comparing the immunogenicity of FVIII product classes (pdFVIII and rFVIII products)[37]. The study, which included 251 previously untreated patients (PUPs) with severe hemophilia, showed that treatment with pdFVIII containing VWF resulted in a significantly lower incidence of inhibitors than treatment with rFVIII (26.8% versus 44.5%). Although there are some questions regarding the quality of the study design and the generalizability of the findings to Europe and North-America (as most of the patients were from Egypt, India and Iran) for so far this is the only randomized controlled trial which showed that rFVIII products almost double the risk of inhibitor formation compared to pdFVIII. The debate is however still ongoing. More details regarding the possible protective role of VWF will be provided later on in this article (see ‘Predictors of ITI outcome’).

All known factors contributing to the formation of inhibitors are summarized below, as proposed by van Helden et al (**figure 2**)[46]. In this model each patient has an individual threshold for developing inhibitors, determined by genetic factors. Subsequent environmental factors, like intensity of treatment at first exposure, product type and administration of FVIII in the absence or presence of inflammation,

determines whether sufficient immune activation occurs to exceed the threshold for inhibitor formation. This simplified model provides a better understanding of the complexity of the immune response to FVIII and also explains why some patients do and other patients do not develop inhibitors.

3.3 Immune response to FVIII

Allo-immunization to infused FVIII follows the classical immune response paradigm, which can be divided in the primary and secondary response[20,47] (**figure 3**). The primary immune response starts with recognition and endocytosis of the infused FVIII by antigen presenting cells (APCs), most notably DCs but also macrophages and B-cells. Thereafter FVIII is presented as FVIII peptide/major histocompatibility complex (MHC) class II complexes to naïve T-cells in the lymph node[20]. In the presence of an activating, pro-inflammatory micro-environment these T-cells become activated and differentiate into FVIII-specific CD4+ T-cells. Subsequently, the FVIII-specific T-cells provide B-cell help, allowing B-cells to undergo class switching and supporting them to differentiate into FVIII-specific memory B-cells and anti-FVIII producing plasma cells.

There are several requirements for an effective interaction between the APC and the T-cell. This includes the functional avidity of the particular T-cell to bind to the MHC-peptide complex, up-regulation of co-stimulatory signals CD40 and CD80/86 on the APC in the presence of 'danger' signals and pro-inflammatory cytokines. In absence of costimulatory signals, interaction between the APC and the T-cell is aborted and a non-productive immunologic synapse is formed. As a consequence T cells become anergic or undergo apoptosis, which results in antigen specific tolerance instead of a productive immune response[39]. The abovementioned factors may be important factors in the control of tolerance induction in the approximately 2/3 of all patients that do not develop inhibitors to FVIII[47,48].

The secondary response is mediated by FVIII-specific memory B-cells, which act as APCs and activate FVIII-specific CD4+ T-cells. In turn, these T-cells help the memory B-cells to differentiate into antibody secreting plasma cells[23,39,49]. At the same time uptake of FVIII by (other) APCs also stimulates T-cells, resulting in the activation of new FVIII-specific B-cells and thus the generation of additional plasma cells

and memory B-cells. Contrary to the primary immune response, which involves naive T- and B-cells, the secondary immune response to FVIII is mediated by FVIII-specific CD4+ T-cells and memory B-cells. These cells are able to expand response to FVIII much quicker and to produce higher affinity antibodies to FVIII and effective recall responses, all features of effective B-cell memory responses.

4. Immune tolerance induction

4.1 Mechanism of action

ITI is the most widely used therapy to eradicate inhibitors. The exact mechanisms how repeated administration of FVIII re-establishes tolerance remain unresolved, but the general concept of this therapy is that recurrent exposure of the immune system to FVIII under non-inflammatory conditions leads to a down-modulation of the established anti-FVIII antibody response and results in the induction of immune tolerance[48,50].

Since the first description of ITI, many different treatment regimens have been developed, encompassing protocols with variations in FVIII dose, product type and use of additional immunosuppressive agents. The three most well-known protocols are the Bonn protocol, the Van Creveld protocol and the Malmö protocol (**table 2**), but multiple adaptations of these regimens are used.

Table 2. Summary of the Bonn protocol, the Van Creveld protocol and the Malmö protocol.

ITI protocol	Regimen	Success rate	Comments
Bonn protocol[51,52] (high dose)	Start: - FVIII 100-150 IU/kg twice daily - aPCC 50 U/kg twice daily (only for patients at high risk for bleeding) When inhibitor < 1 BU/ml: - FVIII 150 IU/kg once daily	87%	Very demanding for patients High costs
Van Creveld protocol[53,54] (low dose)	- 25-50 IU/kg every second day or 3 times/week	87%	Less demanding Cost saving
Malmö protocol[55]	- Neutralizing continuous infusion of FVIII to maintain FVIII plasma levels > 30% for 10-14d - Cyclophosphamide: 12–15 mg/kg i.v. (days 1–2); 2–3 mg/kg orally (days 3–10) - Intravenous IgG: 2.5–5 g on day 1; 0.4 g/kg/d on days 4–5 - Protein A adsorption: if the inhibitor titre is >10 BU/ml prior to start of therapy to reduce titre to <10 BU/m	59-83%	Rapid response Cost saving Requires hospitalization Concerns regarding use of cyclophosphamide No longer use because long-term responses were not always durable

ITI: Immune Tolerance Induction; FVIII: factor VIII; aPCC: activated prothrombin complex concentrate; BU: Bethesda Units.

Clinical information about the immunologic reactions during ITI in patients remains limited; new knowledge about the physiology of tolerance induction is obtained indirectly, mainly from murine models of hemophilia A. Some differences however exist between human and murine immune responses, such as antibody responses raised against FVIII. Noteworthy herein is the deficiency of IgG4 subclass antibodies in mice[47]. In humans, IgG4 plays an important role in the anti-FVIII immune response as it is one of the most prevalent subclasses of all anti-FVIII antibodies and persistence of this subclass is associated with failure of ITI[23,56]. Results from murine models of ITI therefore need to be interpreted with caution.

Key players of the immune response to FVIII, and therefore also the main targets of ITI, are FVIII-specific CD4+ T-memory cells, FVIII-specific B-memory cells and (long-living) anti-FVIII producing plasma cells. Considering the down-regulation of antigen receptors during the terminal differentiation of plasma cells, it is unlikely that these cells are affected by antigen-specific inhibition or depletion during ITI. Instead it is hypothesized that successful ITI depends on the elimination of FVIII-specific B-memory and CD4+ T-memory cells. Extinction of long-living anti-FVIII producing plasma cells could eventually occur due to the lack of replenishment by B-memory cells. Considering a lifespan up to several years of

long-living plasma cells the abovementioned concept also explains why ITI can take so long to complete[57,58].

But what are the mechanisms that result in inhibition or deletion of FVIII-specific B- and T-cells?

Three mechanisms have been described which all contribute in inducing immune tolerance[48,50] (**figure 4**):

1. Inhibition of B-memory cell differentiation into plasma cells by high FVIII concentrations;
2. Anergy of effector T-cells due to exhaustion / overstimulation and induction of regulatory T-cells due to chronic exposition of FVIII in a non-inflammatory state;
3. Development of anti-idiotypic antibodies: i.e. antibodies directed to anti-FVIII antibodies.

4.1.1 Inhibition of B-memory cells

Exposure to high levels of FVIII inhibits the re-activation of FVIII-specific memory B-cells, preventing them to differentiate into antibody secreting plasma cells[59,60], as was first shown using an *in vitro* and *in vivo* murine model of hemophilia A[59]. In this study CD138-negative splenocytes from mice, which were immunized to human (or murine) FVIII, were analyzed. In these experiments, low concentrations of FVIII (0.01 µg/ml to 0.1 µg/ml), equivalent to about 10-100% of the physiologic plasma concentration of FVIII, stimulated FVIII-specific memory B-cells to differentiate into antibody secreting cells. Supraphysiologic levels of FVIII (1, 10 and 20 µg/ml, i.e. 10 to 200 times the physiologic level of FVIII), however, reduced memory B-cell differentiation and prevented the formations of antibody secreting cells. This inhibition of the B-memory response was irreversible and seemed to involve the activation of caspases, which induce apoptosis. Moreover, it was established that the suppressive activity of high concentration of FVIII was the result of a direct action on FVIII-specific memory B-cells and was not mediated by T-cells.

The abovementioned findings were confirmed in experiments testing a range of concentrations between 1 pg/ml to 100 µg/ml of FVIII in a comparable hemophilia A mice model[60]. Here, the optimal re-stimulation of B-memory cells was seen at concentrations of 3-10 ng/ml (3-10% of the physiological plasma concentration), whereas inhibition started at a level of FVIII of 100-300 ng/ml with an almost

complete inhibition at 1 µg/ml (corresponding to 1-3 times and 10 times the physiological plasma concentration respectively). Of note, the concentration of FVIII required for inhibition was very different between B- and T-cells, namely 100-300 ng/ml and 100 µg/ml, respectively. This finding supports the view that inhibition of FVIII-specific B-memory cells is a T-cell independent process and therefore the selective inhibition and eradication of these B-memory cells might be an early event in the down-modulation of the immune response to FVIII during ITI.

Although these studies provide important insight into a potential mechanism of tolerance induction, it remains unknown whether the process of B-memory cell inhibition occurs in hemophilia patients that undergo ITI. Van Helden et al. showed that the percentage of FVIII-specific B-memory cells in inhibitor patients ranges from 0.05-0.24% of all IgG-producing B-memory cells, while FVIII-specific B-memory cells were absent or present at very low levels in patients successfully treated with ITI[61]. Successful ITI may therefore involve the deletion of FVIII-specific B-memory cells although the mechanism behind the elimination of these cells in humans remains unresolved. Noteworthy in this respect is that the lowest FVIII concentration required to completely suppress FVIII-specific B-cells (1 µg/ml) in the mouse models is higher than the FVIII levels that can be achieved with even the high dose ITI protocol. Moreover, these studies do not explain the clinical efficacy of low-dose ITI protocols.

4.1.2 T-cell anergy and induction of regulatory T-cells

Considering their key role in establishing and maintaining the immune response to FVIII, the adjustment of FVIII-specific CD4+ T-cell function is an essential requirement for the induction of tolerance.

In general, there are several T-cell-dependent mechanisms involved in peripheral tolerance. The most important mechanisms are described below and depicted in **figure 5**[62,63]:

1. Clonal deletion by Fas-mediated activation-induced cell death. In T-cells repeated activation and upregulation of Fas ligand (FasL) results in induction of this pathway of apoptosis. Therefore, Fas-mediated death of T-cells appears particularly important for eliminating lymphocytes that recurrently encounter persistent antigens, most notably self-antigens.

2. Anergy, or functional unresponsiveness of the T helper cell clone: anergy can be caused by presentation of antigen without appropriate co-stimulation or with upregulation of alternative, inhibitory, receptors like CTLA-4 and programmed cell death 1 (PD1);
3. Cytokine-mediated suppression of effector T-cells due to development of regulatory T-cells.

Especially the field of Treg biology has evolved rapidly during the last decades. These Tregs can be divided in two broadly distinct subsets, 'natural' and 'adaptive' Tregs[64]. The natural Tregs, characterized as CD4+CD25+FoxP3+, develop from naive precursors in the thymus and play a role in the maintenance of self-tolerance. In contrast, adaptive Treg develop peripherally without input from the thymus. Adaptive Tregs can develop in case of antigenic stimulation under very specific conditions, including repeated antigen presentation by immature DCs or stromal cells, which express low levels of costimulatory molecules and MHC class II[65]. These adaptive Tregs can be subdivided in Tr1 and Th3 cells, which are characterized by production of the immunosuppressive cytokines IL-10 and transforming growth factor bèta (TGF-β) respectively. The net results are inhibition of T-cell activation and formation of effector T-cells (Teffs), and/or suppression of Teff cell functions.

The chronic exposure of a naive immune system to antigen in the absence of danger-signals leads to the induction of Tregs[66,67]. Thus, the chronic exposure of hemophilia A patients to FVIII under non-dangerous conditions, such as during ITI, could also induce FVIII-specific Tregs, which in turn are able to suppress FVIII-specific Teff cells. Without the help of these Teffs, FVIII-specific B-cells will not differentiate into antibody-producing plasma cells and this will eventually lead to the elimination of these B-cells. Nevertheless, the question remains if the above described scenario would also operate in a primed immune system, which applies to the situation in inhibitor patients[48]. In this condition the immune response to FVIII is regulated by B- and T-memory cells and it might be more challenging to modulate such a pre-existing and optimized inhibitory antibody response[68].

So what is known about the T-cells, and more specific, the Treg response in hemophilia A patients during ITI? As stated before, limited data on phenotypic and functional changes of FVIII-specific lymphocytes during ITI is available, mostly from case studies. For example, T-cell responses of a patient with mild

hemophilia A (missense genotype A2201P) were characterized during one year after initial inhibitor development[69]. The patient developed a high-titer inhibitor (250 BU/ml) that decreased over time to 8 BU/ml. CD25-depleted CD4+ T-cells of this patient showed an enhanced response to an A2201 peptide 11 and 19 weeks after inhibitor detection. Due to the absence of CD4+CD25+ Tregs in these samples, this finding suggests the possible involvement of Tregs in down-regulating immune responses. A possible role of Tregs in inducing tolerance to FVIII was supported by subsequent studies[70]. Since FVIII-specific T-cells are routinely present in normal individuals in absence of clear pathology, Kamaté et al. studied the possibility that naturally occurring Tregs may contribute to the inhibition of the FVIII-specific T-cell response. Therefore the proliferative response against FVIII using unmodified, or Treg-depleted peripheral blood mononuclear cells (PBMCs) was tested in 13 healthy individuals. Depletion of Tregs resulted in a markedly increased FVIII-dependent response. Another study evaluated if eradication of FVIII-specific T-cells could be a possible mechanisms of action in ITI by studying the CD4+ T-cell response to FVIII in five ex-inhibitor patients[71]. Following repeated stimulation with FVIII-loaded autologous DCs, a FVIII-specific T-cell line was detected in one of the five patients, whereas no FVIII-specific T-cells could be isolated from patients without inhibitor or from normal control subjects. Although deletion of FVIII-specific T-cells is a likely mechanism to induce tolerance, this study merely shows that elimination of these antigen-specific T-cells is not necessary to restore tolerance to FVIII. In a study of Hu et al. normal donors and hemophilia A patients with and without inhibitor were analyzed for cytokine production by CD4+ T-cell blasts responding to native FVIII[72]. In normal donors, 23 of the 44 subjects had a significant proliferative response to FVIII. The CD4+ T-cell blasts of these 'responders' produced interferon gamma (IFN-γ) and TGF-β, whereas the CD4+ T-cell blasts of the 'non-responders' produced only TGF-β. Hemophilia patients without inhibitors also produced IFN-γ and TGF-β. In contrast, in patients with inhibitors FVIII exposure induced production of IFN-γ and IL-4, but no production of TGF-β. These findings suggest a role for Th2 cells (illustrated by the IL-4 production) in inhibitor formation, whereas a regulatory Th3 response (illustrated by TGF-β) may prevent antibody synthesis.

In conclusion, most of the knowledge about the role of T-cells in inducing (peripheral) tolerance remains based on fundamental research. Clinical data about the mechanism of ITI in hemophilia is rather limited and is mostly restricted to a cross-sectional comparison of immune profiles between healthy controls and patients with and without inhibitors. However, evidence exists to support that ITI restores immune tolerance to FVIII in part through modulation of pathogenic FVIII-specific CD4+ T-cell responses.

4.1.3 Anti-idiotypic antibodies

Anti-idiotypic antibodies also associate with ITI-induced reestablishment of tolerance to FVIII in hemophilia A patients[73]. It is hypothesized that anti-idiotypic antibodies neutralize potentially damaging auto-reactive antibodies and suppress functions of auto-reactive B-cells[74]. This concept is supported by several findings. First of all both anti-FVIII antibodies and their counteracting anti-idiotypic antibodies are present in healthy individuals[75]. Moreover it was shown that administration of intravascular immunoglobulin (IVIG) in patients with neutralizing antibodies to FVIII can be curative and that this effect was correlated with the presence of anti-idiotypic antibodies in pools of immunoglobulins[76]. Gilles et al. demonstrated in two hemophilia A patients that successful ITI was associated with the formation of anti-idiotypic antibodies, which neutralized the inhibitory capacity of anti-FVIII antibodies[77]. Indeed, plasma from patients treated with ITI, both successful and unsuccessful, contained an anti-FVIII antibody-neutralization factor, whereby this factor was in the IgG fraction and increased during successful ITI[78]. Anti-idiotypic antibodies showed therapeutic potential in a murine hemophilia A model by restoring normal FVIII activity in the presence of a monoclonal inhibitor of the FVIII C2 domain[79]. More recently similar results were found in an *in-vitro* study of anti-idiotypic antibodies directed to human polyclonal anti-FVIII antibodies[73]. Except for their neutralizing capacity of inhibitors, anti-idiotypic antibodies might also suppress FVIII-specific B-cells by crosslinking the B-cell receptor (BCR) with the inhibitory FcγRIIB (CD32) receptor on these cells[80]. The FcγRIIb receptor contains an immunoreceptor tyrosine-based inhibitory motif (ITIM). In the setting of crosslinking of the BCR and FcγRIIb, ITIM-mediated signaling can result in the inhibition of B-cells or the induction of apoptosis[80].

Although the potential role of anti-idiotypic antibodies in inducing tolerance and/or treatment of anti-FVIII antibodies seems promising, the evidence regarding these entities remains limited and clinical tests are lacking.

4.2 Predictors of ITI outcome

Several different studies and registries monitor success rates of ITI to identify predictors of successful outcomes[81–88]. These factors are summarized in **table 3**.

The most consistently recognized predictors of ITI success are related to the strength of the immune response: i.e. the peak historical FVIII inhibitor titer (< 200 Bethesda units (BU)/ml), the inhibitor titer before start of ITI (< 10 BU/ml) and the peak inhibitor titer during ITI. In addition, 'non-null' FVIII mutations (small insertions/deletions and missense mutations) have been associated with a favorable outcome, whereas interruption of ITI seem to decrease the success rate[83,84]. Regarding the domain specificity and IgG profile of anti-FVIII antibodies, van Helden et al. reported that antibodies directed against the light chain of FVIII were associated with a successful outcome of ITI[89], whereas a high proportion of FVIII-specific IgG4 was associated with failure ITI treatment[56]. Although a hemophilic mouse model suggested that stimulation of toll-like receptors (TLR) increased the re-stimulation of FVIII-specific B-memory cells, this association between infection or inflammation and the outcome of ITI has not been reported in humans.

Table 3. Main predictors of Immune Tolerance Induction success[81–88].

	Consistently recognized	Postulated / further data needed	Unlikely
Patient related	Historical peak titer ≤ 200 BU/mL Inhibitor titer < 10 BU/ml at ITI start Peak titer during ITI ≤ 200 BU/ml	Low-risk FVIII genotype Anti-FVIII epitope specificity / IgG subclass	Age at ITI start Time between inhibitor diagnosis and ITI start Ethnicity
Treatment related / environmental factors		FVIII dose* Type of FVIII product Interruptions of ITI Infections / other immunological challenges	

* Poor risk patients: better prognosis with high-dose regimen; good risk patients: no difference in efficacy between high- and low-dose regimen, although high-dose regimen associated with shorter duration to success and less bleeding complications.

Probably the two most discussed issues regarding predictors of ITI are the FVIII dose and product type. Concerning the optimal FVIII dose, the International Immune Tolerance Registry (IITR) and the North-America Immune Tolerance Registry (NAITR) showed conflicting results[81,82]. A meta-analysis of these two registries demonstrated that in good-risk patients, defined as an historical peak titer < 200 BU/ml and an pre-ITI titer < 10 BU/ml, FVIII dose did not influence outcome, whereas in poor-risk patients, with a historical peak titer > 200 BU/ml and/or pre-ITI titer > 20 BU/ml, greater chances of successful ITI were seen with a daily FVIII dose ≥ 200 BU/ml[90]. In order to clarify the dose issue, the International ITI study was designed[85]. This randomized controlled trial showed that ITI success rate was similar between patients treated with FVIII doses of three times per week 50 IU/kg or daily 200 IU/kg. However, the low dose regimen was associated with a significantly longer time to inhibitor eradication and significantly more bleeding complications, which was one of the reasons for a premature termination of the study[85].

The answer to which FVIII product to use during ITI is even less clear. Traditionally pdFVIII and rFVIII are compared, while since its introduction in ITI, rFVIII-Fc is also included in the discussion.

With regard to pdFVIII versus rFVIII, data suggest that pdFVIII products may be less immunogenic and might result in greater success of ITI compared to rFVIII products[91–93]. The presence of VWF in pdFVIII products was proposed as an explanation for these findings. Possible mechanisms of the protective role of VWF include epitope masking to reduce immunogenicity and prevention of endocytosis by DCs[93–95].

Other factors that could contribute to the reduced immunogenicity of pdFVIII compared to rFVIII include the presence of immunosuppressive molecules, including TGF-β, in plasma derived products and the different posttranslational modification of rFVIII due to its production in mammalian cells rather than human cells[39,96,97].

Clinical evidence to support these hypotheses is mainly originating from historical retrospective cohorts and case series. Kreuz et al. reported a significant decline of ITI success rate from 90% to 29% after introduction of monoclonal pdFVIII or a rFVIII product in patients treated with the otherwise unchanged high-dose Bonn protocol[98]. Moreover, when these patients who failed ITI were switched to pd FVIII, 80% were able to achieve tolerance. Similar findings were reported in patients treated in Bonn and Bremen between 1991 and 2001 (success rate 54% versus >80% for rFVIII and pdFVIII respectively)[99]. However, these uncontrolled retrospective series have some major limitations and bear the risk of being influenced by confounding factors, including that certain patient characteristics might not have been reported and differences in duration of ITI courses: ITI courses with rFVIII were often for shorter duration than ITI courses with pdFVIII.

In 2014 a pooled meta-analysis of 13 studies and 382 patients found no difference in ITI outcome between patients treated with VWF-containing products and patients treated with FVIII products without VWF[100]. However, due to heterogeneity and lack of data of the included studies, a multivariable regression analysis adjusting for baseline risk of ITI failure was not feasible and there seemed to be a higher proportion of poor risk patients in the study population treated with VWF-containing FVIII products. So far, no prospective randomized ITI trial has compared pdFVIII with rFVIII for ITI and no evidence conclusively demonstrates the superiority of any FVIII product, leaving the issue unresolved.

Noteworthy here as well is the role of the extended half-life product rFVIII-Fc in the induction of tolerance. This fusion protein consists of a single molecule of B-domain deleted FVIII and the Fc domain of human IgG1[101,102]. The prolonged half-life of rFVIII-Fc mediated through binding to the neonatal Fc receptor (FcRn), which protects IgG1 and Fc-fusion proteins from lysosomal degradation[101,102]. Except for prolonging the half-life, fusion of FVIII (or other haptens) to the Fc-region of IgG may have additional

immunomodulatory consequences through preferentially inducing Tregs[10,11]. More recently the tolerogenic capacity of rFVIII-Fc has been confirmed by both pre-clinical and clinical studies[13–16]. A mouse model of hemophilia A demonstrated that, compared to B-domain deleted and full length rFVIII, administration of rFVIII-Fc produced significantly lower antibody responses to rFVIII. Hereby it was shown that rFVIII-Fc resulted in up-regulation of Tregs and tolerogenic cytokines and markers, while pro-inflammatory cytokines were down-regulated. Both the interaction of rFVIII-Fc with the FcRn as with Fcγ receptors, of which some are immunosuppressive, such as FcγRIIb, appeared to be involved. Finally, the first case reports of successful ITI with rFVIII-Fc have been published[15,16]. Taken together, these studies about the potential tolerogenic effect of rFVIII-Fc are interesting and promising. More clinical data is needed to establish the role and possibility of added value of rFVIII-Fc in ITI compared to the standard rFVIII products.

5. New mechanistic insights in prevention and treatment of inhibitors

Given the invasive, lengthy and expensive character of the current immune tolerance protocols, much research effort is ongoing to develop novel strategies to eradicate inhibitors. Already in clinical practice, especially in patients who fail ITI, are the (concomitant) use of rituximab and other immunomodulatory drugs like IVIG, cyclophosphamide or mycophenolate mofetil (MMF)[7]. However, all these agents have a general immunosuppressive effect instead of inducing antigen-specific tolerance to factor VIII. This is the focus of several novel strategies, which are mainly directed to the key players of the FVIII immune response and include FVIII-specific B- and T-cell, both Treg and Teff, targeted therapies[47,103,104]. An enumeration of the extensive range of all new experimental approaches falls beyond the scope of this review. However, in **table 4** the most relevant current developments are summarized. Next we will highlight several key strategies. Important to notice here, is that these new strategies mainly showed to be effective in preventing inhibitor formation in a naive immune system instead of extinguishing the anti-FVIII response in a primed immune system.

Considering their essential role in the anti-FVIII immune response, many researchers focus on T-cells as target to induce tolerance, which includes either inhibition of Teffs or induction of Tregs. New

experimental approaches to inhibit Teffs include administration of FVIII combined with anti-CD3 antibodies, mTOR inhibitors or antibodies that block costimulatory pathways (such as inducible T-cell costimulatory (ICOS) and ICOS-L, CD28 and B7 and CD40 and CD40L). Strategies to induce Treg cells include liver-directed gene therapy, orally administered antigen, or the use of engineered FVIII-specific human regulatory T-cells[47,103]. In the latter case, FVIII-specific Tregs were engineered ex vivo by transduction of a T-cell receptor (TCR) isolated from a hemophilia A inhibitor patient T-cell clone into Tregs from healthy human donors[105]. It was shown that these Tregs inhibited the proliferation of FVIII-specific Teffs *in vitro*. Moreover, in a co-culture with splenocytes from FVIII-immunized hemophilia A mice and in the presence of FVIII, the engineered Tregs were able to suppress the generation of anti-FVIII antibody producing cells.

Another interesting effort to induce FVIII tolerance includes the transduction of B-cell blasts to express A2 and C2 IgG heavy chain fusion proteins[12]. These engineered B-cells significantly reduced immune responses to FVIII in naïve hemophilia A mice as well as in previously immunized hemophilia A mice. The effect was shown to persist for at least 2 months and, based on further experiments, is likely dependent on the recruitment of Tregs.

In summary, an extensive array of tolerance induction protocols is emerging in pre-clinical studies. Such protocols have promise to translational efforts in hemophilia A patients but possibly also to patients suffering from autoimmune disorders. What can we learn about immune tolerance restoration that is applicable as possible treatment in autoimmune diseases?

Table 4. Selection of novel strategies for immune tolerance induction in hemophilia A.

Target	Therapy	Mechanism of action	Results
T-cells	Anti-CD3	Prevention of generation of activating APC/T-cell synapse; increase in Treg:Teff ratio	Reduction of inhibitor incidence and anti-FVIII antibody titers in naive hemophilia A mice[106].
	mTOR inhibitors (rapamycin)	Increase in Treg:Teff ratio	Prevention of inhibitor development in naive hemophilia A mice[107].
	Blocking co-stimulatory pathways with antibodies or fusion proteins (anti-ICOS, anti-CD40, CTLA4-Ig)	Induction of CD4+CD25+FoxP3+ Tregs; elimination of Teff in short term; suppression of memory T-cell in long term.	Prevention of inhibitor formation or reduction of inhibitor titers in naive hemophilia A mice; effect on restimulation of FVIII-specific memory B-cells variable; duration of effect also variable[108–111].
	Engineered FVIII-specific Tregs	Suppression of Teff proliferation by antigen-specific Tregs	Decreased generation of anti-FVIII antibody producing cells in immunized hemophilia A mice[105].
B-cells	Antigenic liposomes with CD22 ligand B-cell blasts expressing A2 and C2 IgG fusion proteins	B-cell inhibition by binding to CD22 Treg recruitment	Lower anti-FVIII IgG titers and significant protection from bleeding in naive hemophilia A mice if co-administered with FVIII[112]. Dampened immune response to FVIII in both naive as immunized hemophilia A mice[12].
Plasma cells	Bortezomib	Proteasome inhibitor; non-specific eradication of plasma cells	Significant delay of inhibitor onset if co-administered with FVIII and decrease of anti-FVIII IgG secreting plasma cells in naïve hemophilia A mice. In immunized hemophilia mice only marginally effect of inhibitor progression and no effect on plasma cells[113].
Other	Antigen expression by liver-directed gene therapy (viral vector-mediated)	Generation of transgene specific CD4+CD25+FoxP3+ Tregs, that inhibit antibody formation and CD8+ T cell responses against the transgene products	Vector-dose dependent elimination of pre-existing inhibitors in canine and murine models of hemophilia A (and B) Correction of coagulation (after eradication of inhibitor) due to coagulation factor expression from gene therapy[114,115].
	Oral tolerance induction using transgenic plant cells expressing FVIII antigens (heavy chain and C2 domain)	Induction of different subtypes of Tregs, predominantly LAP+ Treg	Suppression of inhibitor formatting in naïve hemophilia A mice. Tolerance induction in hemophilia A mice with pre-existing inhibitors[116,117].
	Flt3L with rapamycin and FVIII	Flt3L leads to expansion of DC subsets with regulatory properties, resulting in induction of Tregs	Lower inhibitor titers in a naive hemophilia A mice model[118,119].

APC: antigen presenting cell; Treg: regulatory T-cells, Teff: Effector T-cells; ICOS: Inducible T-cell COstimulator; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; Ig: immunoglobulin; Flt3L: FMS-like receptor tyrosine kinase 3 ligand; DC: dendritic cell.

6. Experience with immune tolerance induction in other diseases

Immune tolerance induction is not unique for hemophilia, but can apply to other diseases as well, most notably to allergy and auto-immune disorders (AID) caused by pathogenic antibodies.

Two ITI-like treatments will be discussed in detail and compared to experience in ITI for hemophilia: allergen-based immunotherapy in allergies and an experimental model of multiple sclerosis (MS).

6.1 Immunotherapy in allergy

Allergen-specific immunotherapy (AIT) which can be applied subcutaneously (SCIT) or sublingually (SLIT), serves as an effective treatment to reduce symptoms of allergic asthma and rhinitis and venom-induced anaphylaxis[120]. Here, target antigen is regularly and in increasing dosages administered over a period of months to years. Mechanistically, in AIT a shift from allergen-specific effector T-cells to a regulatory phenotype appears to drive successful outcome in AIT[120–124].

The first phase of AIT is characterized by a rapid desensitization and a fall in degranulation of mast cells and basophils[121,122,124]..

Subsequently early tolerance is achieved by a decline of IL-4 secreting Th2 cell and an induction of IL-10 secreting Treg cells and regulatory B-cells (Breg)[120,123]. The induced Treg cells and their cytokines, mainly IL-10 and TGF-β, subsequently further inhibit Th2-type immune responses and contribute to the control of allergies in different ways. Similarly, induction of IL-10–producing Breg cells inhibits proinflammatory cytokines and supports Treg cell differentiation.

Finally, memory T- and B-cells are responsible for the last phase of tolerance maintenance. Treg cells stimulate the class switching of B-cells towards IgG[120,122]. IgG4 particularly increases during the course of AIT and these antibodies are considered as a classical non-inflammatory isotype[122,124]. First of all IgG4 prevents the release of mediators from mast cells and basophil by competing with IgE for allergen binding. Moreover the IgE receptor may be inhibited due to formation of IgE-allergen-IgG4 complexes, which bind to both the FcγRIIb and FcεRI. Finally IgG4 antibodies are able to exchange Fab arms, leading to unique functional bi-specific monovalent antibodies, and they do not activate complement.

Especially these immunoregulatory function of IgG4 in AIT, or in general, is very interesting, since in hemophilia A IgG4 is one of the most prevalent subtype of antibodies and high levels of IgG4 are associated with failure of ITI[56]. This is contradictory to the physiologic function of IgG4 and needs to be further elucidated.

6.2 Immunotherapy in MS

The auto-inflammatory disorder MS is caused by the aberrant recognition of self-peptides of the myelin sheath and the attack of the central nervous system (CNS) [125]. A well-described animal model for MS is experimental autoimmune encephalomyelitis (EAE), in which T-cells express a transgenic T-cell receptor specific for the immunodominant epitope of myelin basic protein (MBP). In this model immunization with a myelin peptide and an adjuvant triggers EAE, whereas initial administration of the same peptide without adjuvant can successfully prevent EAE[126–128]. Parallel to hemophilia A and allergies, the induction of tolerance in this peptide immunotherapy involves anergy of CD4+ T-cells and a switch in serum cytokines from a dominant interferon-c response towards IL-10[127,129]. Interestingly, a recent EAE study showed that also myeloid-derived suppressor cells (MDSCs) play a role in the process of tolerance restoration and this is described in other autoimmune diseases as well[130–132]. MDSCs are a heterogeneous group of immature myeloid cells with immunoregulatory function, which are extensively studied in the field of cancer for their detrimental role in the immune escape of tumors by suppressing antigen-specific T-cell responses[133]. MDSCs inhibit T-cell functions in several ways, which involve both soluble mediators as well as cell-surface molecules[134]. Wegner et al. studied the role of MDSCs in EAE and they revealed that a subset of MDSCs, known as polymorphonuclear (PMN)-MDSCs, were involved in the generation of tolerance[130]. These cells showed an upregulation of the expression of immunoregulatory markers during peptide immunotherapy and were able to suppress CD4+ T-cell proliferation. This study illustrates that MDSCs are not only important in the pathophysiology of cancer and tumor immune escape, but might be targeted for restoring tolerance in auto-immune diseases as well.

6.3 The influence of antigen dose for the success of ITI

The extent to which chronic antigen exposure leads to Teff anergy or Treg induction appears dependent on antigen dose and -affinity[126]. In the model of EAE it was shown that lower signal strength leads to anergy, while higher signal strength triggers induction of IL-10 secreting Tregs[126]. However, any high-dose peptide-specific therapy can cause a damaging immune response due to the primary burst of cell activation and cytokine release[135]. Therefore, allergen specific immunotherapy typically starts with lower doses, which gradually build up. In hemophilia A, contrary to hemophilia B, anaphylactic or other adverse reactions during ITI are very unusual and there is no build-up phase. Considering the abovementioned dose-dependent differences in T-cell responses combined with the ongoing debate about the optimal FVIII-dose during ITI, an outstanding question remains how exactly FVIII dose might influence the outcome of ITI.

6.4 Other regulatory cells as target for immune tolerance induction

The immunotherapies used in hemophilia A, allergies and MS mainly describe the central role of CD4+ Tregs and to a lesser extent also that of Bregs and MDSCs. However, additional regulatory immune cells may play a role in achieving tolerance, which include (immature) DCs, regulatory macrophages (Mregs), mesenchymal stromal cells and CD8+ T-cells[132]. Data, although predominantly pre-clinical, showing the tolerogenic capacity of each of these cells is growing. A description of known mechanisms of tolerance induction by DCs, Mregs, mesenchymal stromal cells and CD8+ T-cells falls beyond the scope of this review, but has been described elsewhere[132,136–139]. The contribution of these regulatory cell types in tolerance induction to FVIII however remains unknown.

7. Conclusion

ITI is until now the only effective therapy to eradicate inhibitors in hemophilia A. Although knowledge about the mechanisms mediating tolerance induction has expanded significantly, much is still to be elucidated. The hypothesized mechanism involved in ITI is that repeated administration of FVIII in a non-

inflammatory state causes anergy induction of Teffs, induction of Tregs, apoptosis of B-cells and possibly also generation of anti-idiotypic antibodies.

Based on ITI in hemophilia, two important paradigms appear as being essential for the induction of tolerance for other allergies and autoimmune diseases/disorders too. First, antigen should be presented by tolerogenic rather than immunogenic APCs. And secondly, this should result in the induction of regulatory cells. Here, regulatory T-cells and their production of suppressive cytokines, like IL-10 and TGF-β, play a central and pivotal role. However, also other regulatory cells are increasingly identified, which include MDSCs, (immature) DCs, mesenchymal stromal cells, and regulatory macrophages, B- and CD8+ T-cells.

Further research should clarify the exact role of these cells in the process of tolerance induction. Other interesting future research subjects include unraveling the optimal way to direct allergens to a tolerogenic APC, including the role of FVIII dose and type in this process, and elucidating how to eradicate FVIII-specific long-living plasma cells (which are less susceptible to tolerance induction due to downregulated antigen receptors).

8. Practice points

- The development of neutralizing anti-FVIII antibodies currently is the most challenging complication in the treatment of hemophilia A.
- Immune tolerance induction (ITI), consisting of frequent and repeated administration of FVIII, is so far the only proven therapy to eradicate inhibitors.
- Mechanisms involved in introduction of tolerance include Teff anergy and Treg induction, apoptosis of B-cells, and induction of anti-idiotypic antibodies.
- Other regulatory cells, such as (immature) DCs, Bregs and MDSCs, also seem to be involved, although their exact role needs to be further clarified.

9. Research agenda

- Elucidating the mechanism of optimal tolerogenic antigen (FVIII) presentation, including the influence of FVIII dose and product type, but also co-administration of immunomodulators such as anti-CD3 and the blockade of costimulatory pathways.
- Defining the role of less known regulatory cells during ITI, such as Bregs, MDSCs or mesenchymal stromal cells.
- Research on how to specifically target allergen-specific long-living plasma cells during ITI.

Disclosure

The authors report no conflicts of interest.

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Figure legends

Fig. 1. FVIII and VWF complex.
Schematic model of von Willebrand Factor (VWF) and factor VIII (FVIII) complex in plasma. One subunit of the multimere VWF is shown. The heterodimer FVIII consists of a heavy chain and a light chain, which are non-covalently bounded to each other (dotted lines between A1-A3 and A2-A3). By its C1 and A3 domains FVIII is also non-covalently bounded to the D'/D3 domains of VWF[94,140,141].

Fig. 2. Model of inhibitor formation.
Several genetic factors, i.e. factor VIII (FVIII) genotype, polymorphisms in immune response genes and MHC class II type, determine the threshold or the susceptibility of inhibitor development. Additional environmental factors, including intensity of treatment and immune system challenges such as infections or surgery, determine if the threshold is reached and if inhibitors will develop or not.
Adapted from Helden et al., Haemophilia[46]. Reproduced with permission from the publisher.

Fig. 3. Primary and secondary immune response to FVIII[20,47].
I. Primary immunization: Upon initial exposure to factor VIII (FVIII), the protein is internalized by antigen presenting cells (APCs), e.g. dendritic cells (DCs), and is presented to naive CD4+ T-cells. Together with the presence of an activating, pro-inflammatory micro-environment with upregulation of co-stimulatory signals, this results in activation of the T-cell. In turn, these activated T-cells activate FVIII-specific naive B-cells, which expand and differentiate either into plasma cells, secreting anti-FVIII IgM antibodies (FVIII plasma cell), or FVIII-specific B-memory cells (FVIII B-mem).
II. Secondary immunization: During the secondary immune response, FVIII B-mem act as APCs and activate FVIII-specific T-memory cells (FVIII T-mem). After this interaction and activation FVIII B-mem will further differentiate into anti-FVIII IgG secreting plasma cells.
MHC: Major histocompatibility complex; CD40L: CD40 ligand; TCR: T-cell receptor.

Fig. 4. Proposed working mechanisms of ITI.
Repeated administration of factor VIII (FVIII) in a non-inflammatory state leads to presentation of FVIII without costimulatory signals (A) and/or upregulation of inhibitory T-cell molecules (B), such as CTLA-4 or PD1. This in turn causes anergy of FVIII-specific effector T-cells (Teffs) and induction of regulatory T-cells (Tregs), indicated by the balance shifting towards the Treg site. These Tregs exhibit inhibitory effect on both T- and B-cells. At the same moment high doses of FVIII induce apoptosis of FVIII-specific B-memory cells (FVIII B-mem). The elimination of these FVIII B-mems reduces antigen presentation and subsequent activation of FVIII T-mem, which further shifts the balance from Teff towards Treg. The net result of these events is that (long-living) plasma cells are not replenished, which eventually leads to the eradication of inhibitors. Finally also the development of anti-idiotypic anti-FVIII antibodies might be involved in this process by neutralizing the effect of anti-FVIII inhibitors.

Fig. 5. T-cell dependent mechanisms of peripheral tolerance[62,63].
I. A normal T cell response is characterized by activation of T-cells due to the recognition of antigen in the presence of costimulatory signals.
II. Encounter with (auto-)antigen might induce T-cell anergy if costimulatory signals are absent or inhibitory T-cell molecules, such as CTLA-4, are upregulated.
III. Self-reactive T-cells might be eliminated after contact with (auto-)antigen by activation-induced cell death due to upregulation of T-cell FasL and interaction with death receptor Fas.
IV. Suppression of Teff duo to the effect of Treg and their inhibitory cytokines.
APC: antigen presenting cell; CTLA-4: cytotoxic T-lymphocyte associated antigen 4; FasL: Fas ligand; Teff: effector T-cells; Treg: regulatory T-cells.

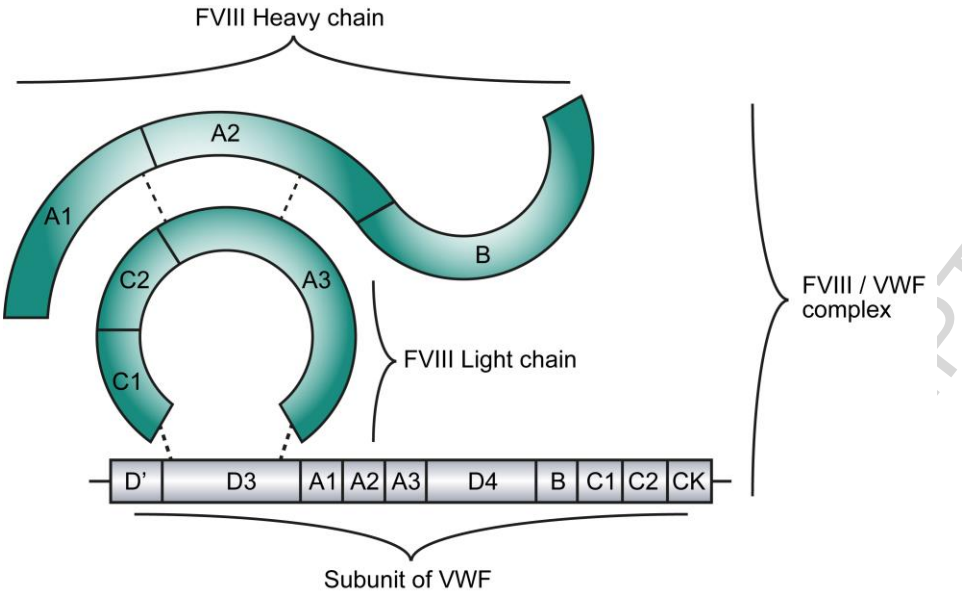

Fig. 1

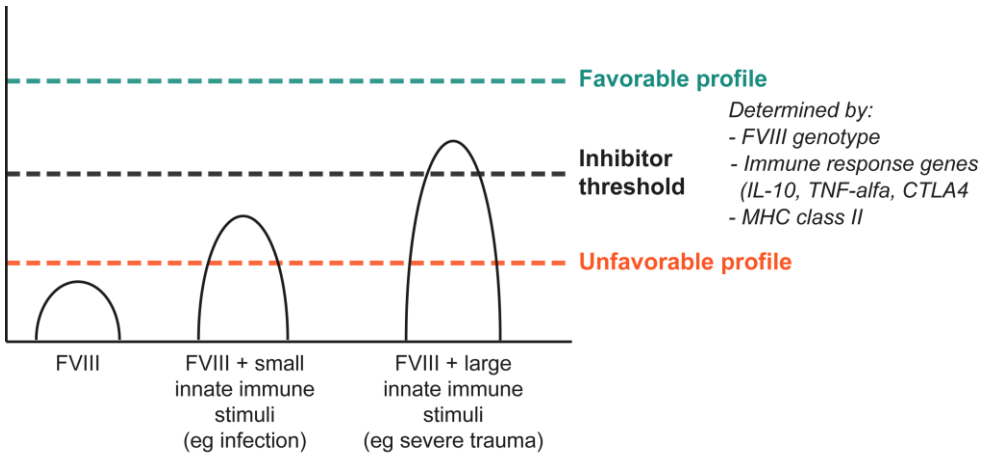

Fig. 2

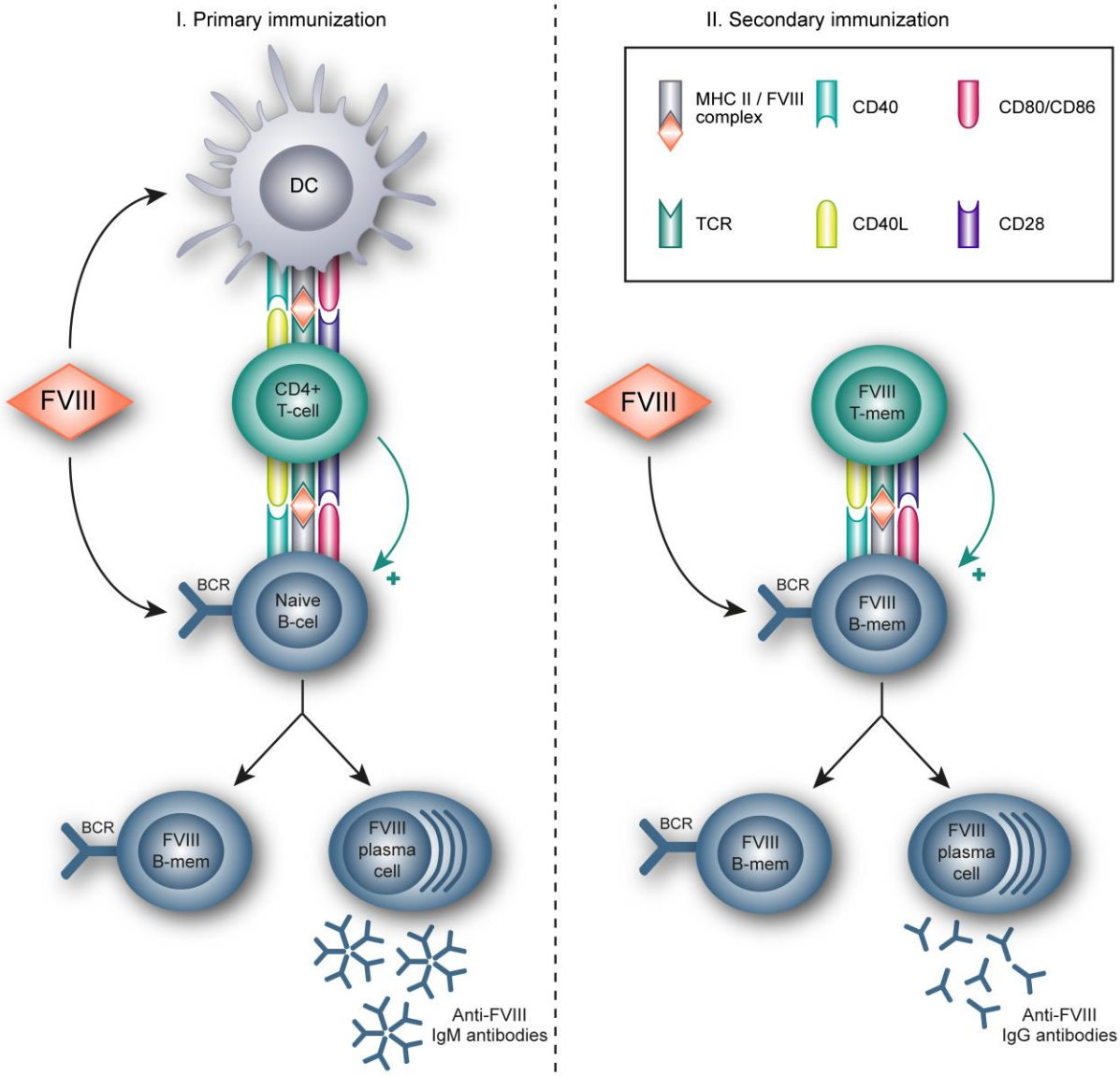

Fig. 3

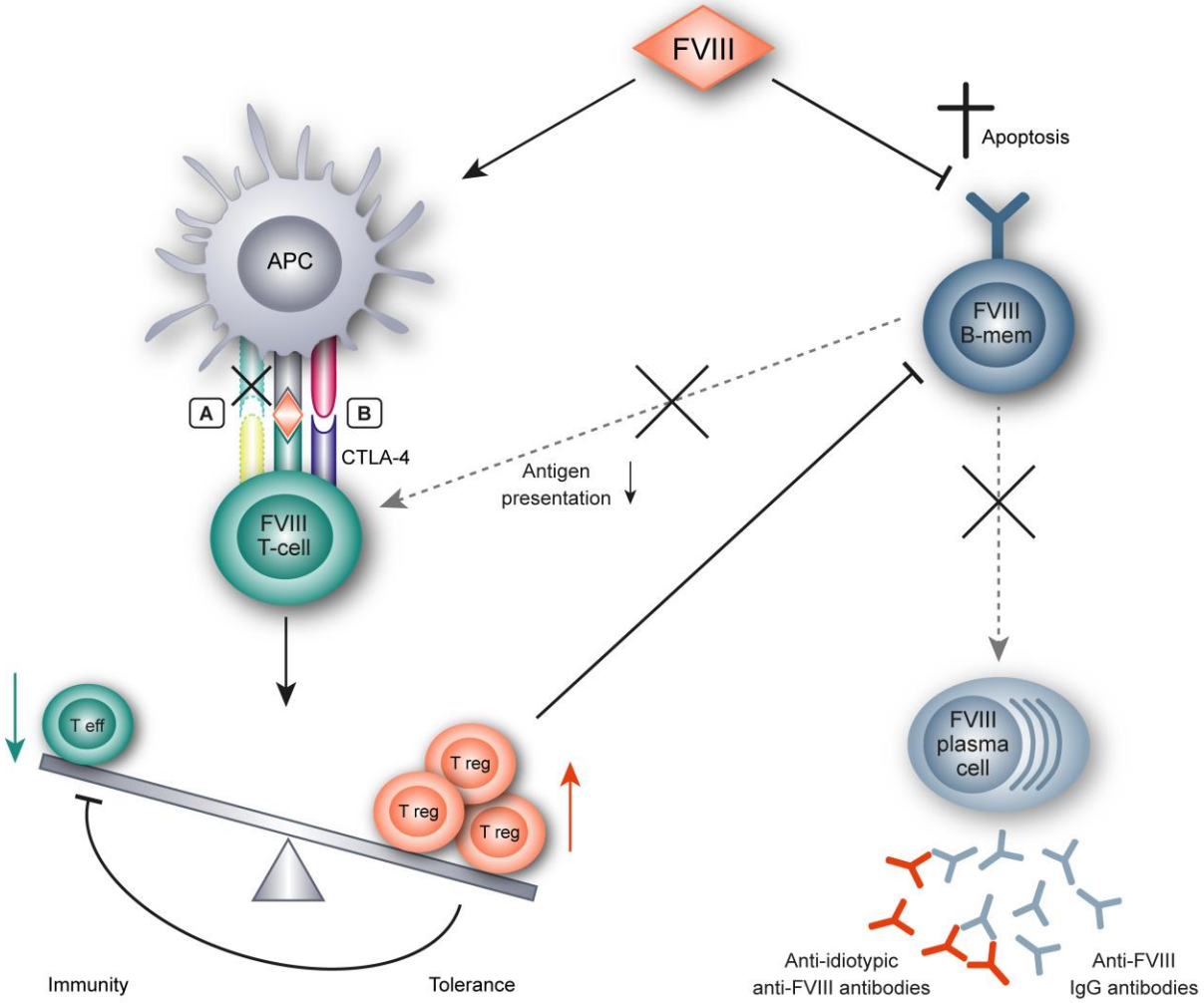

Fig. 4

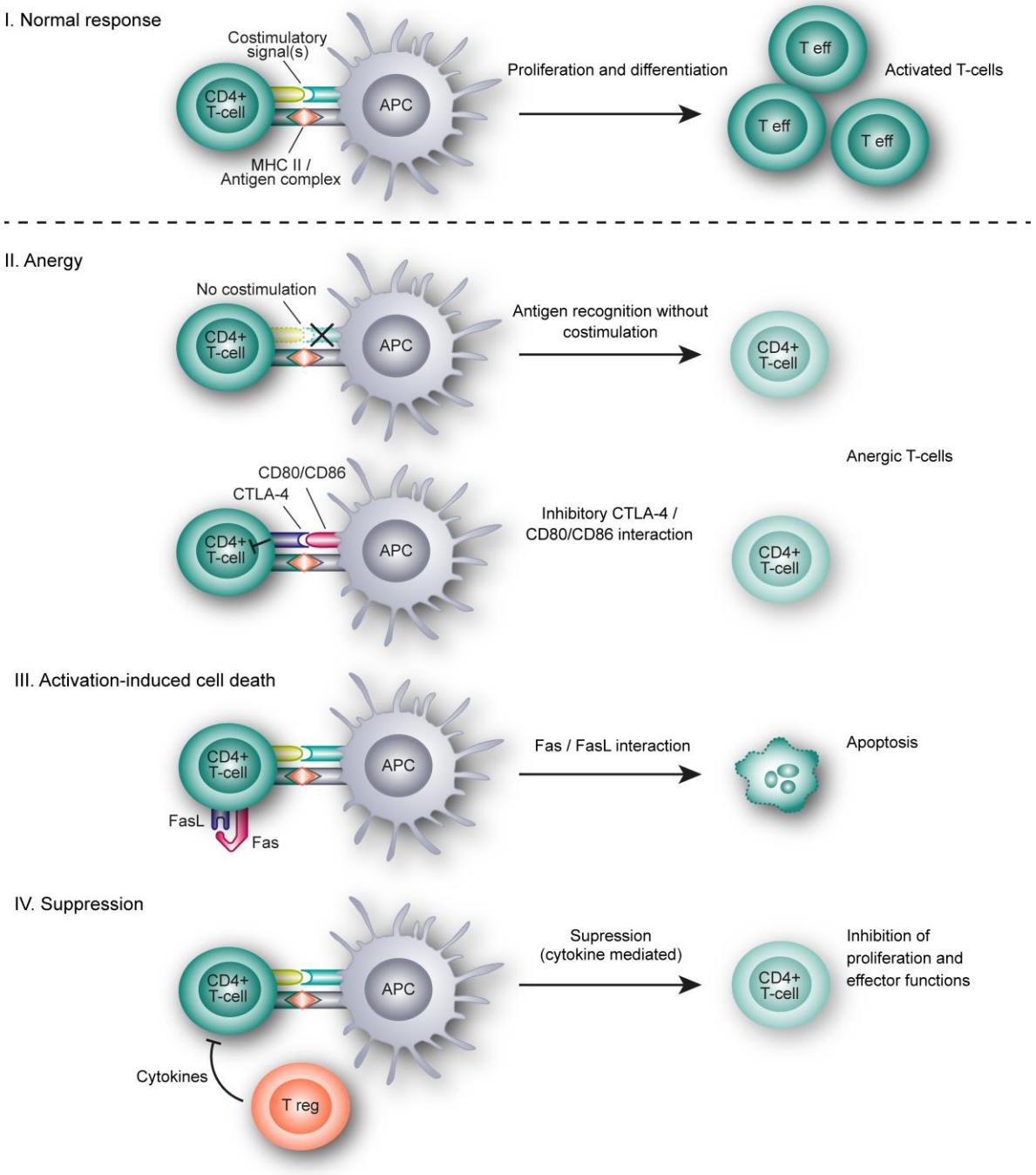

Fig. 5