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Inhibitor formation in congenital haemophilia A: an immunological perspective

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Abstract

The immunogenicity of therapeutic factor VIII (FVIII) in patients with hemophilia A has been puzzling scientific and clinical communities for more than 3 decades. Indeed, the development of inhibitory antibodies to FVIII remains a major clinical challenge and is associated with enormous societal costs. Thus, the reasons for which a presumably innocuous, short-lived, intravenously administered glycoprotein triggers such a deleterious, long-lasting neutralizing immune response is an enigma. This review does not pretend to bring an answer to this challenging question. It will however summarize the latest findings regarding the molecular interactions at play in the recognition of FVIII by the immune cells, the validity of the proposed risk factors for FVIII allo-immunization, and the different solutions that allow induction of FVIII-specific tolerance in preclinical models of hemophilia A.

Introduction

Haemophilia A (HA) is an X-linked inherited disorder caused by a defect in the gene encoding coagulation factor VIII (FVIII). The disease is typically divided in three categories of severity based on the circulating levels of pro-coagulant FVIII detected in plasma. Haemophilia A is “mild” (5-40% of residual FVIII compared to normal plasma), “moderate” (1-5%) or “severe” (<1%). The most efficient strategy to correct or prevent bleeding in the patients relies on the intravenous administration of exogenous therapeutic FVIII that is either plasma-derived (pdFVIII) or recombinant (rFVIII). Currently, the most serious complication of FVIII replacement therapy is the development of anti-FVIII antibodies with neutralizing properties that are referred to as “FVIII inhibitors”. While anti-drug antibodies typically arise in 1-5% of the patients, FVIII inhibitors develop in up to 30% of the patients with HA.¹ In addition, the intravenous route of administration is classically considered as non-immunogenic.² The elevated rate of development of inhibitory antibodies to FVIII is thus intriguing from an immunological stand-point. Here, we will review the current knowledge on the anti-FVIII immune response with a specific focus on the molecular interactions at play in the recognition of FVIII by the cells of the innate and adaptive immune system and on the intuitively proposed risk factors for FVIII allo-immunization. The review will also propose a short summary of the different immuno-intervention approaches validated in preclinical models of HA to confer tolerance to exogenous FVIII.

Dynamics of the anti-FVIII immune response

The primary immune response to therapeutic FVIII

The development of FVIII inhibitors results from a classical allogenic immune response that is dependent on CD4 T cells.³ The implication of T cells has been suggested empirically in inhibitor-positive patients who became tolerant to exogenous FVIII following HIV infection.⁴

It was further suggested by the lack of anamnestic immune response to exogenous FVIII upon treatment using antagonist anti-CD40-ligand (CD40L) antibodies.⁵ The mandatory step for initiation of a T cell response is antigen recognition and internalization by a professional antigen presenting cells (APCs). After endocytosis, FVIII is cleaved into small peptides that associate with molecules of the major histocompatibility complex of class II (MHC class II) depending on their affinity. The APCs then migrate to secondary lymphoid organs such as spleen or lymph nodes to reach T cell-rich areas. Complexes of FVIII-derived peptide and MHC class II molecules are exposed at the surface of APCs and are presented to naïve CD4 T cells. The recognition of FVIII-derived peptide by the T-cell receptor (TCR) of T lymphocytes provides a first activation signal (signal 1). Additional signalization implicating interactions of ligands and receptors at the surface of T cells and APCs (signal 2), and presence of soluble pro-inflammatory stimuli such as cytokines (signal 3) are also required in the micro-environment to fully activate the FVIII-specific naïve T cells. Such signals involve at least interactions between CD40L expressed at the T cell surface with CD40 exposed on the APC membrane,^{6,7} as well as CD86 and CD28 expressed by APCs and T cells, respectively (Figure 1).⁸ Once activated, T cells migrate towards the B cell follicles. In general, humoral responses directed against proteins involve the follicular B cell pathway within secondary lymphoid organs.⁹ The early phase of this activation pathway corresponds to an extra-follicular response where naïve B cells that have internalized the antigen through their membrane immunoglobulin or B-cell receptor (BCR) present CMH II/peptide complexes to activated T cells. The formation of the T/B cell synapse triggers intracellular signaling that leads to the differentiation of follicular B cells into short-lived plasma cells that produce IgM or IgG of low affinity.^{9,10} During the late phase of the follicular B cell response, activated B cells re-enter the follicles to interact with follicular helper T cells and form germinal centers (GCs).⁹ GCs are specialized micro-environment for T and B cell interactions leading to the

proliferation/selection of B cells and accumulation of somatic hypermutations in the genes encoding the variable regions of the BCRs. GCs responses generate memory B cells that are able to quickly differentiate into antibody secreting cells (ASCs) after re-exposition to the antigen.¹¹ GCs associated responses also generate long-lived plasma cells.¹² In the case of anti-FVIII immune responses, the presence of low affinity anti-FVIII antibodies has been documented in healthy donors, **non-exposed patients to exogenous FVIII** and inhibitor-negative patients.^{13,14} It is conceivable that the production of such low affinity antibodies results from early extra-follicular responses. In contrast, the finding of high affinity anti-FVIII IgG4 observed in inhibitor-positive patients implicates the differentiation of follicular B cells involving formation of GCs and help from FVIII-specific follicular T cells.^{13–15}

The secondary immune response to therapeutic FVIII

Primary responses involve naïve FVIII-specific T and B cells. In contrast, subsequent exposures of the organism to exogenous FVIII recruits FVIII-specific memory B cells generated during the primary immune response. Memory B cells are able to develop a faster and stronger immune response against FVIII than naïve B cells, and their recruitment leads to the production of high affinity neutralizing antibodies.^{10,14} T and B cell interactions are also essential during secondary anti-FVIII humoral responses in HA mice. The administration of an anti-CD40L monoclonal antibody to FVIII-deficient mice that have already developed a primary immune response to FVIII leads to GCs depletion after 24 hours. In addition, the repeated injection of FVIII concomitantly with an anti-CD40L antibody prevents the formation of new GCs and the production of anti-FVIII antibodies, while it does not allow induction of long-term tolerance.⁵ Thus, CD40-CD40L interactions play a crucial role in formation and maintenance of GCs during the secondary immune response to FVIII. Anti-FVIII memory B cells are essentials to maintain long-term humoral response and to drive

quick anamnestic response upon FVIII re-exposure. While memory B cells are preferentially located in lymphoid organs, Van Helden *et al* successfully detected circulating FVIII-specific B cells in inhibitor-positive HA patients. By incubating irradiated-murine thymoma EL4B5, that express human CD40L with CD19-positive B cells from patients during 9 to 10 days, they allowed the *in vitro* differentiation of memory B cells into ASCs. The presence of FVIII-specific memory B cells was then tested by ELISpot assay.¹⁶ The frequency of circulating FVIII-specific memory B cells represented 0.05% to 0.24% of surface IgG-positive B cells. Importantly, the secondary immune response generates long-lived plasma cells that are likely to migrate to specific niches in the bone marrow.^{9,17} In FVIII-deficient mice, FVIII-specific plasma cells are detected in the bone-marrow from the third injection of FVIII where they survive for more than 22 weeks without any further FVIII injection.¹⁸

Initiation of an anti-FVIII immune response

Antigen presenting cells

Different types of APCs may be involved in the capture and endocytosis of therapeutic FVIII in patients. Among these, dendritic cells (DCs), macrophages, and B cells are the most probable candidates. The type of APCs at play depends on the immune status of the patients towards FVIII, in particular whether their organism has previously been exposed to exogenous FVIII or not. In previously untreated patients (PUPs), where the immune system never encountered FVIII, a role for naïve FVIII-specific B cells is very unlikely owing to their very low frequency. Recently, Zerra *et al* observed in FVIII-deficient mice the co-localization of exogenous FVIII in the marginal zone of the spleen at the level of a specialized B cell subset, marginal zone (MZ) B cells. MZ B cells express polyreactive B cell receptor and are located at the interface of blood circulation and immune system. The authors showed that the depletion of MZ B cells leads to a significant reduction of the anti-FVIII immune response.¹⁹

This observation may reflect the role of MZ B cells in trafficking FVIII from the MZ to B-cell follicles and to the CD4 T-cell and DC rich areas, rather than a direct role of MZ B cells in priming of naïve T cells.²⁰ Besides, Archambault *et al* demonstrated, using a mouse model of conditional expression of MHC class II, that B cells are less prone than DCs to present antigens to naïve T cells during primary immune response.²¹

Macrophages are generally described as highly efficient phagocytic cells due to the expression of a myriad of endocytic receptors. In blood, macrophages circulate as monocytes and differentiate into macrophages after infiltrating tissues. Several specialized sub-populations of macrophages are present in the spleen.²² Interestingly, thirty minutes after intravenous injection, our seminal work had shown the co-localization of FVIII mostly with metallophillic and MZ macrophages in the MZ of the spleen of hemophilic mice. Depletion of splenic macrophages and CD11c⁺/CD8 α ⁻ DCs by injection of clodronate-containing liposomes abrogated the anti-FVIII humoral response.²³ As mentioned in the case of MZ B cells, this observation may reflect the role of MZ macrophages in cellular and molecular exchange between the MZ and the white pulp of the spleen in the initiation of antigen-specific immune responses.²⁴ Of note, the ability of macrophages to express co-stimulation molecules and activate naïve CD4 T cells is lower than that of DCs.^{25,26}

It is important to mention that techniques used to date to validate the role of different splenic APC subsets rely primarily on the physical elimination of the cells, either by targeting cells characterized with a high endocytosis capacity using clodronate-containing liposomes or using depleting antibodies that target particular APC populations. Such approaches are not without consequences on the architecture of the MZ of the spleen²⁷ and interpretation on whether the targeted APC subtype is at play in the initiation of the anti-FVIII immune response or whether the disrupted MZ splenic structure is an artefactual reason for the reduced onset of FVIII-specific immune responses is not clear.

DCs are often described as ‘sentinel’ cells²⁸ by virtue of their high plasticity. DCs circulate in the blood in an immature state, and several distinct sub-populations of DCs are present in different tissues. DCs are able to sample antigens and to simultaneously detect danger signals. Following their migration to secondary lymphoid organs, they orchestrate the outcome of T-cell presentation: depending on the nature of signals they sense in the micro-environment, they may promote T-cell inactivation and induction of immune tolerance or, on the contrary, T-cell priming and activation. Because of the central role of DCs in presenting antigen to naïve T cells, our group and others have used human immature monocytes-derived DCs (MO-DCs), an *in vitro* source of DCs, to investigate the capture and presentation of FVIII by APCs. MO-DCs are able to endocytose FVIII and to present FVIII-derived peptide to human FVIII-specific T cell lines.^{29–31}

Endocytic receptors for FVIII

Antigens may be internalized via three different ways: macropinocytosis, phagocytosis or receptors-dependent endocytosis. Macropinocytosis consists in the internalization of large quantities of soluble antigen by non-specific membrane invagination. On the basis of the low concentration of circulating FVIII, it is unlikely that FVIII is internalized by APCs through macropinocytosis. Phagocytosis consists in the capture of large soluble particles such as virus, bacteria or apoptotic cells. In the case of protein antigens, endocytosis occurs mostly by means of receptors. Receptors-dependent endocytosis is very efficient from a kinetic point of view and implicates defined motives on the glycoproteins; this allows the capture of antigens present at low concentrations in the surrounding environment.²⁶ Since FVIII circulates mostly as a complex with its chaperon molecule von Willebrand factor (VWF), the internalization of FVIII may occur through endocytic receptors that bind VWF or FVIII itself. Indeed, several endocytic receptors for VWF and FVIII have been identified.^{32,33} The endocytosis of FVIII or

the FVIII/VWF complex may lead to FVIII accumulation in lysosomes followed by its degradation, or to the trafficking of FVIII to the endosomal compartments and presentation of FVIII-derived peptide/MHC class II complex at the cell surface. The outcome of FVIII (catabolism or presentation to T cells) following its uptake depends on the site of internalization, the nature of APCs and the receptors that are involved in endocytosis. Currently, endocytic receptors that are known to bind to VWF and FVIII have been mostly involved in the clearance of VWF and/or FVIII. This review will focus mainly on endocytic receptors that have been shown to bind to FVIII (Table 1).

Low density lipoprotein receptor family

The role of the low-density lipoprotein-1 (LRP-1 or CD91) in FVIII catabolism has been documented in several studies.^{34,35} The physiological relevance of LRP-1 in FVIII clearance was demonstrated in a mouse model in which deletion of LRP-1 increased the endogenous plasma FVIII levels and FVIII half-life.³⁶ Furthermore, epidemiological studies in human of two polymorphisms of the LRP-1-encoding gene have shown that this receptor modulates the levels of plasma FVIII.³⁷ FVIII presents three potential binding sites to LRP-1: a low affinity site located in the C2 domain³⁴, and two high affinity sites situated in the A2 and A3 domains.^{38,39} Under physiological conditions, the binding region for LRP-1 on the heavy chain (A2 domain) is only exposed after proteolytic cleavage of FVIII.⁴⁰ Thus, while intact FVIII only interacts with LRP-1 via its light chain, activated FVIII binds to LRP-1 through both its heavy and light chains. When VWF is complexed to FVIII, it interferes with the binding site on the light chain.³⁴ In a scenario proposed by Sarafanov, the VWF/FVIII complex binds to heparan sulfate proteoglycans (HSPG), a component of the extracellular matrix, leading to the concentration of FVIII at the cell surface and dissociation of the VWF/FVIII complex, thus exposing the binding site on FVIII for LRP-1.⁴¹ More recently,

Rastegarlarlari *et al* observed a direct interaction of VWF with LRP-1 at the surface of macrophages under shear stress conditions, thus highlighting another possible mechanism of binding of VWF/FVIII complex to LRP-1.⁴² While LRP-1 has been mostly involved in the catabolism of antigens, several studies have reported a role of LRP-1 in the development of immune response.^{43,44} Indeed, the binding of heat shock proteins (HSP) and proteins released by tumor cells to LRP-1 on APCs leads to their internalization and activation of nuclear factor kappa B (NF- κ B) pathway, subsequently leading to the maturation of APCs and the secretion of pro-inflammatory cytokines and allowing the polarization of the T-cell response toward Th1 and Th17 profiles.^{43,45} Others receptors of the LDL-R family may contribute to FVIII uptake by APCs, since LDL-R interacts with FVIII and regulates the circulating FVIII levels in concert with LRP-1.^{46,47} Yet, a role for LRP-1 or others LDL-R in the initiation of anti-FVIII immune response remains to be demonstrated, since an involvement of these receptors in FVIII uptake by MO-DCs has been excluded by several studies.^{31,48,49}

Asialoglycoprotein receptor

The scavenger receptor asialoglycoprotein receptor (ASGPR) was reported to recognize β -D-galactose and N-acetyl-galactosamine residues that are predominantly exposed on the B-domain of FVIII.⁵⁰ Because no obvious differences in the half-life and immunogenicity of full-length FVIII and B domain deleted FVIII (BDD-FVIII) have been observed, the role of this receptor in FVIII catabolism or endocytosis by APCs remains to be determined.⁵¹ In agreement with this, the use of galactose to saturate ASGPR had no effect on FVIII uptake by DCs.²⁹

Siglec-5

Recently, Pegon *et al* reported that sialic-acid binding immunoglobulin-like lectins-5 (Siglec-5), a human receptor of the lectin family, binds to both VWF and FVIII. Siglec-5 recognizes sialic acid-ending polysaccharide chains. While binding of VWF to Siglec-5 is dependent on sialic acid exposure, binding of FVIII is not. Interaction of FVIII and VWF with Siglec-5 expressed at the surface of HEK293 cells leads to endocytosis of both proteins and accumulation in early endosomes. The over-expression of human Siglec-5 in mice induces the decrease in plasma levels of VWF and FVIII, suggesting that Siglec-5 contributes to the regulation of circulating levels of VWF/FVIII in human.⁵² Since, human MO-DCs and plasmacytoid DCs express Siglec-5, further investigations deciphering the role of this receptor in FVIII uptake and processing by human DCs would be interesting.⁵³

The macrophage mannose receptor or CD206

To date, the only endocytic receptor that has been shown to lead to FVIII processing and presentation to CD4 T cells is the macrophage mannose receptor (MMR) also known as CD206.⁵⁴ Pre-incubation of MO-DCs with mannan, an antagonist of mannose-sensitive receptors, inhibits more than 50% of FVIII endocytosis, and abrogates the activation of a FVIII-specific T-cell line.^{29,55} CD206 is a divalent ion-dependent endocytic receptor that belongs to the C-type lectin receptor family. The tissue distribution of CD206 is well documented in mice, but remains poorly known in human.⁵⁶ In human, CD206 is expressed by different macrophages and dendritic cells subsets as well as by hepatic, splenic, lymphatic and dermal microvascular endothelial cells.⁵⁶⁻⁵⁸ CD206 is involved in innate immune responses through its capacity to recognize a wide variety of pathogens (Gram-positive or Gram-negative bacteria, yeasts, parasites and mycobacteria).⁵⁹ It also plays a role in adaptive immune responses and leads to antigen presentation.⁶⁰ Moreover, CD206 is involved in the modulation of the catabolism of many endogenous glycoproteins in mice.⁶¹ FVIII is heavily

glycosylated and harbors 25 potential sites of glycosylations, 20 of which are glycosylated.^{62,63} rFVIII and pdFVIII present similar glycosylation profiles, including high mannose structures, with bi-, tri- or tetra-antennary complex type chains, that are located on Asn239 and on Asn2118 in the A1 and C1 domain, respectively.^{62,64} While Asn239 carries either high-mannose, or hybrid or complex structures, Asn2118 present only high mannose structures.⁶⁵ Glycans at Asn239 and at Asn2118 are implicated in FVIII binding to CD206. However, since CD206 has not been involved in FVIII endocytosis by mouse bone-marrow derived-dendritic cells, the precise role of CD206 in FVIII endocytosis and presentation to T cells *in vivo* remains to be demonstrated.⁴⁹

CLEC4M

More recently, another C-type lectin receptor, CLEC4M, also known as CD299, L-SIGN or DC-SIGNR, was reported to bind, internalize and catabolise VWF and FVIII, either in their complex form or not.^{32,66} CLEC4M is expressed by sinusoidal endothelial cells in the liver, placenta, lymph nodes, lung and gastro-intestine tractus.⁶⁷ As in the case of CD206, the binding of CLEC4M is dependent on high mannose oligosaccharides exposed on FVIII. Since CLEC4M has been mostly described as an adhesion receptor, a direct role of CLEC4M in FVIII immunogenicity is unlikely.

FVIII residues involved in endocytosis

Several attempts have been made to identify the key residues of FVIII implicated in its internalization by APCs, independently from any preconceived idea on the nature of the endocytosis receptors at play. Using the monoclonal antibody KM33 that recognizes an epitope located in the C1 domain of FVIII, Herczenik *et al* have demonstrated that the Arg2090, Lys2092 and Phe2093 residues are involved in FVIII endocytosis by MO-DCs.³¹

The substitution of these residues to alanines by site directed mutagenesis generated a FVIII mutant that is significantly less internalized by APCs.^{68,69} More recently, using a similar approach with a monoclonal anti-C2 antibody, we demonstrated that Arg2215 and Arg2220 of the C2 domain also participate in FVIII endocytosis by MO-DCs.⁶⁸ The substitution of these residues to alanine residues yielded a mutant FVIII that was less prone to endocytosis by MO-DCs. Mutation of the three C1 residues showed conflicting results on the immunogenicity of FVIII in FVIII-deficient mice.^{68,69} Taken together, the available data demonstrate the involvement of complex interaction networks that modulate FVIII uptake and lead either to FVIII catabolism or processing and presentation to T cells.

Facilitation of endocytosis of FVIII by other molecules

The experimental set-up used to study the endocytosis of FVIII by APCs generally leaves aside key proteins that may be directly or indirectly implicated in the internalization process. This is true for VWF and complement molecules which are virtually absent in serum free conditions, or are inactivated or present in low amounts when 10% decompemented fetal calf serum is used. Our seminal work had documented a significant reduction of FVIII endocytosis by MO-DCs in the presence of VWF.^{49,70} VWF was thus found to inhibit the binding of FVIII to CD206.^{29,55} Recent finding by the Voorberg lab have provided a more complexed picture of the role of VWF on FVIII endocytosis and processing by APCs. Sorvillo *et al* thus demonstrated that, while most of VWF remains bound to the cell membrane, FVIII-derived peptides are still detected in association with MHC class II molecules at the surface of MO-DCs.⁷¹ The comparison of the MHC II-bound FVIII peptidome when FVIII was incubated alone or with VWF in the presence of MO-DCs showed changes in the repertoire of FVIII-derived peptides presented on HLA-DR molecules.⁷¹ Interestingly, VWF-derived peptides were detected in association with HLA-DR molecules only when VWF was incubated with

FVIII, suggesting that the presence of FVIII mediates VWF endocytosis. Currently, it is not clear whether the same endocytic pathways are involved when FVIII is complexed to VWF or not. Importantly, the fact that VWF was found to be endocytosed in a LRP-1-dependent manner by macrophages under shear-stress conditions, should encourage new investigations of FVIII/VWF uptake by MO-DCs under non-static conditions. The repercussion of VWF co-endocytosis with FVIII on the onset of the anti-FVIII immune response *in vivo* is difficult to predict. It has been hypothesized that the concomitant presentation of VWF-derived peptides by APCs may recruit VWF-specific regulatory T cells that would down-regulate the activation of naïve FVIII-specific T cells and thus favor tolerance to therapeutic exogenous FVIII.⁷² This could explain at least in part the proposed protective effect played by VWF on the immunogenicity of plasma-derived FVIII.⁷³

Molecules of the complement system play major roles in the development of innate and adaptive immune responses. Our group recently investigated the role of complement system on FVIII endocytosis and presentation to T cells by MO-DCs. We observed that the C3 component of the complement system and its cleavage product C3b increase the endocytosis of FVIII by MO-DCs and its presentation to a FVIII-specific CD4 T-cell hybridoma (Rayes et al in press). The interaction of C3b with FVIII was demonstrated by Elisa, and FVIII and C3b were found to co-localize on the surface of MO-DCs. Interestingly, complement activation restored the endocytosis of the C1 mutant (Arg2090Ala-Lys2092Ala-Phe2093Ala) by MO-DCs. The transient depletion of C3 using humanized cobra venom factor resulted in a drastic reduction of the immune response to exogenous FVIII in FVIII-deficient mice (Rayes et al in press). Taken together, these observations highlight new endocytic route(s) for FVIII which are dependent on interactions between FVIII and C3b.

The outcome of FVIII presentation by APCs: T cell activation or tolerance induction?

Danger signals/environmental risk factors

According to the danger signal theory proposed by Matzinger,⁷⁴ the presence of danger signals in the micro-environment at the bleeding site, by promoting the maturation of APCs, is required to initiate anti-FVIII immune responses. Indeed, as explained above, naïve FVIII-specific CD4 T cells can be activated only if they received signal 1 (MHC II/FVIII peptide triggering the TCR), signal 2 (co-stimulatory molecules) and signal 3 (cytokines) from APCs. Failure to provide signals 2 and 3 turns the naïve T cells into regulatory T cells. Danger signals are sensed by APCs through pattern recognition receptors (PRR). Danger signals may be of endogenous origin, such as molecules released upon cellular lesion or cellular stress, or exogenous, such as pathogen-associated molecular patterns (PAMP). Depending on the presence of such signals in the micro-environment, immune system will either initiate a neutralizing immune response or, on the contrary, will activate regulatory mechanisms. Currently, the nature of the danger signal(s) implicated in the anti-FVIII immune response remains unknown. Pfistershammer *et al* demonstrated that *in vitro* FVIII is not able to induce MO-DCs maturation.⁷⁵ Accordingly, using HEK293 cell lines transfected with Toll-like receptors (TLR), a family of PRR, we reported that FVIII alone is not able to activate TLR pathway.⁷⁶ More recently, Miller *et al* observed a synergistic effect of pdFVIII and lipopolysaccharide (LPS) on the expression of the co-stimulatory molecules CD83 and CD86 on MO-DCs. It is thus possible that pdFVIII plays a synergistic role on DC maturation in the context of inflammation.⁷⁷ However, the absence of an effect of rFVIII in the same experimental setup suggests that the synergistic effect of pdFVIII is induced by molecules contained in the pdFVIII product and not by the very FVIII molecule. In addition, while several clinical and pre-clinical data reported a higher risk to develop FVIII inhibitors in patients treated with rFVIII as compared to patients treated with pdFVIII, no study reported a higher risk in patients treated with pdFVIII.

The pro-coagulant activity of FVIII may provide danger signal, since FVIII injection in a context of bleeding in HA patients activates the FX that accumulated at site of bleeding which leads to a burst of thrombin generation.⁷⁸ Skupsky *et al* demonstrated that heat-inactivated FVIII is less immunogenic than native FVIII, and that inhibition of thrombin by hirudin at the time of FVIII administration significantly reduces the anti-FVIII immune response in FVIII-deficient mice.⁷⁹ In contrast with this finding, Meeks *et al* demonstrated the similar immunogenicity of wild-type FVIII and of an inactive Val634Met FVIII variant (which can be activated by thrombin but lacks co-factor activity) and an inactive Arg372Ala/Arg1689Ala FVIII variant (which cannot be cleaved by thrombin and does not dissociate from VWF).⁸⁰ Furthermore, we observed that the inactive Val634Met FVIII mutant is still immunogenic in FVIII-deficient mice in the absence of activation of the extrinsic coagulation cascade pathway.⁸¹ Thus, whether the pro-coagulant activity of FVIII is a source of the danger signal that triggers the anti-FVIII immune response remains unclear.

The inflammatory state of the patients has often been proposed as a potential risk factor to develop FVIII inhibitors. FVIII injection during an infection/surgery or at the time of vaccination have been proposed to increase the risk to develop inhibitors owing to the presence of exogenous or endogenous pro-inflammatory signals.

The hypothesis was strengthened by a pilot study conducted in 26 PUPs treated by early prophylaxis, started at the time of the first bleedings and using lower frequencies and lower doses of FVIII than standard treatment (once 25 IU/kg/week versus thrice 40-50IU/kg/week for the standard protocol). During the first 20 exposure days, FVIII injections were strictly performed at distance of possible inflammatory events such as infections, surgery or vaccinations.⁸² The incidence of FVIII inhibitors was significantly reduced compared to an historical control group (48% vs 3.8% study group). These data suggest that injection of exogenous FVIII in a context that avoids danger signals (bleeding, infection or immunization,

etc...) decreases the risk for allo-immunization. This finding was however not reproduced in another cohort of patients,⁸³ and studies in preclinical models of hemophilia A yielded conflicting results. While induction of an acute bleeding in the knee joint of FVIII-deficient mice did not increase the anti-FVIII immune response,⁸⁴ bleeding episodes in hemophilic rats increased FVIII immunogenicity.⁸⁵ As far as vaccination is concerned, a retrospective analysis on 375 PUPS on the influence of vaccination on inhibitor development, reported the absence of association between vaccination and FVIII exposure.⁸⁶ Similarly, in humanized hemophilic mice, the injection of vaccinal antigens before FVIII administration decreased the frequency of immunization to exogenous FVIII.⁸⁷

Several studies reported that intensive FVIII treatment in case of severe hemorrhages or surgery is associated with a higher prevalence of FVIII inhibitor development.^{88,89} As evoked earlier, this could be explained by alert signals inherent to these events, but also to the exposure of the organism to high amounts of FVIII antigens. Indeed, intuitively, the dose of antigen administered is predicted to affect the intensity of the induced immune response. This was demonstrated in the case of FVIII in earlier studies by Reipert *et al* wherein the intravenous administration of 0.1 or 1 µg rFVIII to FVIII-deficient mice lead to 10-fold different levels of anti-FVIII IgG.⁹⁰

Interestingly, the use of prophylaxis in HA patients was correlated with a lower risk to develop inhibitors.^{82,88,89} In principle, prophylaxis, as opposed to on-demand treatment, consists in injecting lower amounts of FVIII at regular intervals independently from the occurrence of bleeding events, to provide an as continuous as possible exposure of the organism to the exogenous FVIII antigen. In line with the antigen discontinuity theory proposed by T Pradeu, such a continuous exposure to an exogenous antigen should prevent the onset of neutralizing immune responses and favor the induction of a peripheral tolerance.⁹¹

Genetic risk factors for the development of an inhibitory anti-FVIII immune response

Among genetic risk factors, the mutation of the *F8* gene leading to HA has been established. Thus, mutations resulting in severe alterations of FVIII expression such as the inversion of intron 22, large deletions and nonsense mutations significantly increase the risk to develop inhibitors to therapeutic FVIII.⁹² Such mutations lead to the expression of a strongly truncated FVIII or to the complete absence of endogenous FVIII (intron 22 inversion) and the patients with these mutations are referred to as “cross-reactive material” (CRM) negative. From an immunological point of view, the lack of expression of endogenous FVIII in CRM-negative patients results in the absence of thymic education of newly arising T cells and lack of establishment of central tolerance: in absence of FVIII expression in the thymus, FVIII-reactive CD4 T cells are not eliminated during the process of negative selection, and FVIII-specific regulatory T cells (Tregs) are not induced. In contrast, CRM-positive patients have higher chance to develop central tolerance to FVIII and hence to control the immune response to exogenous FVIII. The majority of CRM-positive patients generally produce an endogenous FVIII with a missense point mutation, that is either lacking pro-coagulant activity (e.g., FVIII with the V634M mutation),⁹³ or that accumulates in the endoplasmic reticulum and is poorly secreted.^{94,95} We and others have documented the importance of the affinity for HLA-DR of the peptides from wild-type therapeutic FVIII that correspond to the peptides of endogenous FVIII that carry the HA-causing mutation, as a risk factor for allo-immunization to therapeutic FVIII.^{96–98} Yet, despite the fact that tolerance is not perfect when a mutated FVIII is expressed, the risk to develop FVIII inhibitors is drastically reduced in CRM-positive patients. Gouw *et al* in their meta-analysis on 5283 patients with severe HA from 30 different cohorts observed a higher risk in patients with large deletions or nonsense mutations as compared to patient with intron 22 inversions (INV22).⁹⁹ Although somewhat surprising, those results could be explained by the intracellular expression of two FVIII polypeptides

(FVIII_{I221} et FVIII_B) in peripheral blood cells (PBMCs) and hepatic tissue from patients with INV22.¹⁰⁰ The authors proposed that the two FVIII polypeptides may be expressed by medullary thymic epithelial cells and favor the induction of T-cell tolerance to FVIII. The latter hypothesis remains to be confirmed in view of the fact that another study failed to detect FVIII-encoding mRNA in PBMCs from INV22 patients.¹⁰¹ Presumably, patients who do not develop a central tolerance to FVIII, rely on the induction of peripheral tolerance in order to control of FVIII-reactive T cells.

Importantly, all CRM-negative patients do not develop inhibitors since inhibitors are detected in roughly 30% of patients with severe HA. The mechanisms involved in the induction of peripheral T-cell tolerance to FVIII CRM-negative patients and in the subsequent control of the anti-FVIII immune response have not been elucidated as yet. Investigations of the genetic risk factors associated with FVIII inhibitor development suggest the involvement of multiple factors. Polymorphisms in different immune genes, including IL-10, TNF- α and CTLA-4 genes, have been associated with the onset of FVIII inhibitors.^{102–104} In particular, polymorphisms associated with high IL-10 and TNF α production levels were more frequent among inhibitor-positive patients.^{105,106} Of note, IL-10 was first described to down-regulate the synthesis of Th1 cytokines and was associated with polarization of immune responses towards a Th2 type which are characterized by isotype switch and increased antibody-mediated immune responses. Hu *et al* reported that proliferating FVIII-specific CD4 T cells exhibit different phenotypes depending on the immune status of the patients. While T-cell blasts generated from inhibitor-positive patients were polarized towards both the Th2 (positive for the interleukin IL-4) and Th1 (positive for the cytokine IFN- γ) pathways, T-cell blasts obtained from inhibitor-negative patients were of Th1 and Th3 (TGF- β -positive) type. Interestingly, FVIII-specific T cell blasts isolated from healthy donors were polarized either towards the Th1 or Th3 pathways, but never towards Th2.¹⁰⁷ The authors proposed that Th1

cells may mostly be implicated in the initiation of anti-FVIII immune response, whereas polarization towards a Th2 response may favor the development of a strong immune response to FVIII with the production of FVIII inhibitors. However, the underlying mechanisms are probably more complex since IL-10, a typical Th2-type cytokine that is also the hallmark of Tregs, is produced by monocytes and is endowed with pleiotropic effects in immunoregulation and inflammation. For example, IL-10 down-regulates the expression of MHC class II and costimulatory molecules at the surface of macrophages, while promoting B-cell survival and proliferation. The ABO blood groups were recently proposed to modulate FVIII immunogenicity. Thus, Franchini *et al* reported in a retrospective study on a small cohort of severe hemophilia A that non-O blood group patients have a higher risk to develop inhibitors, independently from other covariates (FVIII products, *F8* mutation).¹⁰⁸ To date, ABO blood groups have mostly been reported to influence VWF and FVIII clearance.¹⁰⁹ The mechanisms underlying the reduced immunogenicity of FVIII in blood group O patients remain to be determined. Recent investigations involving the genome-wide association of 13,331 single nucleotides polymorphisms (SNPs) from 1,081 immune genes in 833 patients with severe HA¹¹⁰ have revealed additional polymorphisms in multiple genes involved in immune regulation. Thirteen SNPs were significantly associated with inhibitor risk, 5 SNPs being correlated with an increase prevalence (MAPK9, DOCK2, CD44, IQGAP2 and CSF1R), and 8 SNPs being apparently protective (PDGFRB, PCGF2, HSP90B1, F13A1, IGSF2, ALOX5AP, MAP2K4 and PTPRN2).

Of note, we have reported the increased prevalence of short tandem GT repeats in the promoter of the gene encoding heme-oxygenase 1 (HO-1) in patients who have developed inhibitory antibodies to FVIII.¹¹¹ HO-1 catalyses heme to generate the anti-inflammatory and immuno-suppressive compounds carbon monoxide and biliverdin, and has been associated with Treg functions.^{112,113} Recently, another regulatory enzyme that also supports Treg

functions was implicated in the control of the anti-FVIII immune response: indoleamine 2,3-dioxygenase 1 (IDO1).¹¹⁴ IDO1 is a heme-enzyme involved in the metabolism of tryptophan and is expressed by various cell types including DCs. Tryptophan metabolites resulting from IDO1 activity promote immune tolerance by inducing the apoptosis of T cells and promoting the differentiation of Tregs.¹¹⁵ Interestingly, PBMCs isolated from inhibitor-positive HA patients failed to express IDO1 and anti-inflammatory cytokines upon *in vitro* TLR9 stimulation in contrast to PBMCs from inhibitor-negative patients. Interestingly, in FVIII-deficient mice, the induction of HMOX-1 and of IDO1 were independently found to protect from the development of a humoral response to FVIII.^{114,116} Taken together, these observations suggest the existence of mechanisms of peripheral induction of T-cell tolerance to FVIII that are deficient in patients with FVIII inhibitors.

Therapeutic strategies to prevent and control the anti-FVIII immune response

Modification of FVIII structure

The endocytosis of FVIII being a prerequisite for the onset of the anti-FVIII immune response, a strategy consists in modifying the structure of FVIII in order to prevent its recognition by APCs. The modification of residues involved in FVIII endocytosis by DCs at the level of the C1 or C2 domains as well as Asn2118 was shown to prevent the uptake of FVIII by human DCs but did not abrogate the onset of the anti-FVIII immune response in FVIII-deficient mice.^{68,117} Moreover, designing less immunogenic FVIII molecules by introducing mutations may be hardly compatible with the preservation of its structure and pro-coagulant activity. An alternative to the traditional approach of site directed mutagenesis has recently been explored to generate better expressed and possibly less immunogenic FVIII variants. Using ancestral sequence reconstruction (ASR), Zakas *et al* have recently identified two common FVIII-encoding ancestor genes: An53 from the rodent lineage and An68 from a

lineage common to primates and rodents, that share 95 and 87% homology with human FVIII sequence, respectively.¹¹⁸ Recombinant BDD An53 and An68 were more efficiently produced and secreted than human BDD-FVIII. Interestingly, the two ancestor FVIII molecules were also less antigenic than BDD-FVIII as they were poorly recognized and neutralized by a panel of anti-A2 and anti-C2 monoclonal antibodies. Further investigations on T-cell epitope prediction as well as immunogenicity in preclinical models of HA are needed to determine whether ASR is a viable strategy to design less immunogenic FVIII in the future.

Immuno-intervention

Prevention of HLA-DR mediated interactions between APCs and T cells

A strategy to prevent the initiation of the anti-FVIII immune response consists in preventing the signalization required for the priming of naive FVIII-specific T cells by targeting signal 1 (Table 2). Using *in silico* measurement of the binding of FVIII-derived peptide to MHC II molecules, Moise *et al* predicted key modifications of major immuno-dominant T-cell epitopes at the level of the C2 domain. The substitutions drastically decreased the affinity of FVIII peptides affinity for MHC II as well as subsequently T-cell activation.¹¹⁹ Although theoretically attractive, such a ‘de-immunization’ strategy of FVIII appears risky and highly complex in view of the polyclonality of the anti-FVIII immune response and involvement of a large number of T-cell epitopes largely spread over the FVIII structure, and in view of the complexity of introducing point mutations in FVIII without affecting its pro-coagulant activity and ability of being intracellularly trafficked and secreted, as evoked earlier.¹²⁰

Inhibition of B cells

B cells are the precursors of plasma cells that secrete anti-FVIII antibodies. Several strategies for targeting B cells have been tested, including the use of proteasome inhibitors.¹⁷ One promising strategy is based on the use of liposomal nanoparticles coated with FVIII and the CD22 ligand (CD22L).¹²¹ Stimulation of the BCR in association with CD22 signalization down regulates B cells in an antigen-specific manner.¹²² Indeed, in FVIII-deficient mice, a single administration of FVIII and CD22L complexed to liposomes nanoparticles reduced the production of inhibitory anti-FVIII IgG. However, the latter strategy has not been tested in the context of repeated injections of FVIII which would be necessary to evaluate the induction of tolerance to FVIII. Our yet unpublished work demonstrates that blocking the BCR signalization pathway using inhibitors of the Bruton's tyrosine kinase does not prevent the onset of a naive anti-FVIII immune response in mice, but reduces the memory response to therapeutic FVIII in FVIII-challenged mice, thus paving the way to strategies aimed at ameliorating the outcome of immune tolerance induction by administration of high dose FVIII.

Induction of T-cell tolerance

Drug-mediated immunosuppression has been tested with success in preclinical models of HA. Thus, the daily *per os* administration to FVIII-deficient mice of rapamycin, an inhibitor of the IL-2 signalization pathway, concomitantly with repeated injections of FVIII was associated with a significant decrease in the anti-FVIII humoral response.¹²³ Rapamycin treatment favored the emergence of CD4⁺CD25⁺Foxp3⁺ regulatory T cells and down-regulated Th2 polarization. Rapamycin-mediated immunosuppression was however not antigen-specific. Recent investigations using synthetic nanoparticles containing rapamycin and FVIII gave promising results in hemophilic mice.¹²⁴ A very brief low dose regimen of methotrexate has

been recently shown to promote tolerance to the human therapeutic enzyme alglucosidase alfa in a mouse model of Pompe disease. Methotrexate-induced tolerance involved the induction of regulatory B cells that secrete IL-10 and TGF- β .¹²⁵ The effect of methotrexate in dampening FVIII immunogenicity remains to be tested.

Another alternative approach to favor the expansion of Tregs *in vivo* consists in the use of IL-2/anti-IL-2 antibody (JES6-1) complexes.¹²⁶ The administration of this complex in parallel to FVIII treatment during 4 weeks was found to inhibit the production of anti-FVIII antibodies in mice, and to induce tolerance in the following weeks. The protective effect was associated with a 7-fold increase in the number of circulating natural CD4+CD25+Foxp3+Helios Tregs, as compared to a control group. The Tregs were however short-lived, highly suppressive but probably not specific for FVIII. In parallel, FVIII-specific human regulatory T cells engineered by retroviral transduction of polyclonal Tregs from healthy donors with an anti-FVIII TCR coding sequence was shown to down-regulate anti-FVIII T and B cell responses *in vitro*.¹²⁷ In a different strategy, our group has shown that the injection to pregnant FVIII-deficient mice with the A2 and C2 domains of FVIII fused to the murin Fc γ 1 fragment, is followed by the transplacental transfer of A2-Fc γ 1 and C2-Fc γ 1 to the foetuses. The transfer was dependent on the neonatal Fc receptor and induced central as well as peripheral T-cell tolerance that lasted for several weeks after birth.¹²⁸

Lastly, the induction of FVIII-specific tolerance upon administration of FVIII by the oral route has been investigated. The latter strategy exploits the tolerogenic environment that characterizes the gut-associated lymphoid tissue. The proof of concept was brought with the oral administration of the purified C2 domain of FVIII to FVIII-deficient mice, either by the nasal or oral routes, prior to subcutaneous injection of the C2 domain with adjuvant.¹²⁹ However, induction of tolerance using this protocol requires enormous quantities of FVIII and the maintenance of FVIII integrity until it reaches the gut. In an alternative attempt, bio-

encapsulated FVIII fragments fused to the B subunit of the cholera toxin (CTB), a transmucosal carrier, allowed the absorption of FVIII fragments by gut microbial flora follow gavage. The treated mice demonstrated a drastically reduced humoral anti-FVIII response.¹³⁰

Conclusion

The present days are facing a paradoxical situation where a myriad of different approaches for immuno-intervention and induction of specific tolerance to FVIII are being validated in hemophilic mice, and where novel therapies using bypassing agents or gene therapy may render the administration of exogenous FVIII **no longer the standard of care** in the future. At the same time, the knowledge we have of the elevated immunogenicity of FVIII in HA patients remains very incomplete and fragmented. Advances in our understanding of the way FVIII is captured and internalized by APCs, of the nature of the dominant T and B cell epitopes, of the major genetic risk factors as well as in the validation or refutation of intuitively proposed danger signals are however undeniable. From both a scientific and clinical points of views, we strongly believe that deciphering the immunogenicity of FVIII should remain a priority for the community. Importantly, the experience accumulated in the field of FVIII and hemophilia A shall lead the way in the more general context of immunogenicity of therapeutic proteins.

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Legends to figures

Figure 1¹. Initiation of the anti-FVIII immune response. Therapeutic exogenous FVIII is captured by APCs that cleave FVIII into small peptides in the endosomal compartment. FVIII-derived peptides associate with molecules of MHC class II. Complexes of FVIII-derived peptide and MHC class II molecules are exposed at the surface of APCs and are presented to naïve CD4 T cells. The recognition of FVIII-derived peptide by the TCR of the T lymphocytes provides the first activation signal (signal 1), interactions of co-stimulation molecules and ligands at the surface of T cells and APCs provide a second signal (signal 2), a third signal is mediated by soluble pro-inflammatory stimuli such as cytokines (signal 3). The three signals are required to get the full activation of naïve T cells and differentiation into effector T cells. Naïve B cells that have internalized FVIII through their BCR, present CMH II/peptide complexes to the activated FVIII-specific T cells. The formation of the T/B cell synapse triggers intracellular signaling that leads to the differentiation of follicular B cells either into memory B cells or plasma cells.

¹ Abbreviations: PAMPS, pathogen-associated molecular patterns; TFs, tissue-factors; PRR, pattern recognition receptors; TCR, T-cell receptor.

Table 1. Endocytic receptors for FVIII²

Receptor	FVIII binding	Catabolism	Endocytosis leading to peptide presentation*	References
LRP-1	Light chain (inhibited by VWF) A2/A3 domain of activated FVIII	Yes	No	34,37–40,48
LDL-R	Specific site unknown	Yes (in mice)	No	36,47
HSPG	A2 domain	Yes in mice (LRP-1 dependent and LRP-1 independent)	No	41
ASGPR	Desialylated B-domain associated glycans	Yes (in mice)	No	50,51
Siglec-5	Specific site unknown	Yes (in mice expressing human Siglec-5)	unknown	52
CD206 (MMR)	Exposed mannose glycans at Asn239 and Asn2118 (inhibited by VWF)	No (in mice)	Yes	29,55
CLEC4M	High mannose glycans	No	No	66,131,132

* In *in vitro* model using mouse or human DCs

² Abbreviations: LRP-1, low-density lipoprotein related receptor-1; LDL-R, low-density lipoprotein receptor; HSPG, heparan sulfate proteoglycan; ASGPR, asialoglycoprotein receptor; Siglec-5, sialic-acid binding immunoglobulin-like lectin-5; MMR, macrophage mannose receptor; CLEC4M, C-type lectin domain family 4 member M.

Table 2. Therapeutic strategy to prevent the development of inhibitors in preclinical model³

Target	Therapeutic strategy	Mechanism of action	FVIII-specific	References
APCs (Endocytosis of FVIII)	Modification of FVIII structure	Prevention of FVIII capture by APCs	Yes	69,81
T cells/ APCs synapse (signal 1)	Prediction of T cell epitope and design of mutated FVIII	De-immunization by prevention of HLA-DR mediated interactions	Yes	119
B cells	FVIII and CD22L coated liposomal nanoparticles	Suppression of B cell response by engagement of CD22	Yes	121
Tolerance induction	Rapamycin	Inhibition of Il-2 signalization, emergence of regulatory T cells	No/ Yes, if formulated in FVIII containing nanoparticles	123,124
	Methotrexate (Single low dose regimen)	Induction of Il-10 and TGF- β secreting regulatory B cells	Not tested in preclinical model of HA (described as antigen specific)	125
	Il-2/ anti-Il-2 complexes	Emergence of Tregs	No	126
	Materno-foetal transfer of A2/C2 domain of FVIII fused to Fc fragment of IgG1	Induction of central and peripheral tolerance by passive transfer of A2 and C2 from the mother to the foetus	Yes	128
	Administration of bio-encapsulated FVIII fragments fused to the B subunit of the CTB	Induction of tolerance through mucosal route, emergence of Tregs	Yes	130

³ Abbreviations: APCs, antigen-presenting cells; HLA-DR, Human Leukocyte Antigen – antigen D Related; CD22, cluster of differentiation-22; Il-2, interleukin-2; Il-10, interleukin-10; TGF- β , transforming growth factor beta; Tregs, regulatory T cells; Fc, Fc- Fragment of IgG; CTB, Cholera toxin B.