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Review Article

Molecular Mechanisms of Inhibitor Development in Hemophilia

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Abstract. The development of neutralizing antibodies in hemophilia is a serious complication of factor replacement therapy. These antibodies, also known as "inhibitors", significantly increase morbidity within the hemophilia population and lower the quality of life for these patients. People with severe hemophilia A have an overall 25-40% lifetime risk of inhibitor development, compared to that of 5-15% lifetime risk in those with moderate/mild hemophilia A. The risk is lower in hemophilia B population (about 1-5%) and occurrence of inhibitors is almost only seen in patients with severe hemophilia B. The understanding of the pathophysiological mechanism leading to the development of inhibitors in patients with hemophilia has improved considerably over the last 2 decades. Identification of early biomarkers which predict inhibitor development in previously untreated patients with hemophilia will assist in risk identification and possible early intervention strategies. In this review, we aim to summarize the molecular mechanisms of inhibitor development in hemophilia and to identify potential areas in need of further investigation.

Keywords: Inhibitors; Hemophilia; Anti-FVIII antibodies; Anti-FIX antibodies.

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Introduction. Hemophilia A (factor VIII deficiency) is an X-linked, recessive bleeding disorder due to the deficiency of coagulation factor, and it is estimated to affect 1 in 5,000 live male births.[1] Hemophilia A is about four times more common than hemophilia B (characterized by factor IX deficiency). The severity of the disease is classified based on the residual amount of functional clotting factor measured in plasma, with persons with <1% factor defined as severe; 1-5% as moderate; and >5%-<40%, as mild.[2] Although clinical trials involving gene therapy are currently ongoing, there is no available cure for hemophilia yet. Current treatments require lifelong, frequent, intravenous infusions of expensive clotting factor protein that are manufactured from human plasma or through recombinant DNA technology.

Moreover, about 30% of severe hemophilia A patients and 5% of severe hemophilia B patients on replacement therapy develop an immune response to the exogenous protein. The development of neutralizing antibodies in hemophilia is a severe complication of factor replacement therapy. Antibodies that neutralize the procoagulant function of factors are known as inhibitors. The incidence of inhibitor development reflects the severity of the molecular defect: FVIII inhibitors develop in 20% to 35% of patients with severe hemophilia A and in 3% to 13% of mild/moderate patients.[3-5] Immune tolerance to factors

has been a major concern and interest for many years because the development of inhibitors significantly increase morbidity and lower the quality of life within the hemophilia population. While hematologists and immunologists have developed and tested a myriad of different drugs and techniques in animal model of hemophilia, current treatments available to by-pass inhibitors in patients are few, variable in their effectiveness, and extremely expensive.[6] Different risk factors have been proposed to be associated with inhibitor development. These include risk factors associated with the type of preparation of therapeutic FVIII (i.e., either the plasmatic or recombinant origin of FVIII), with the inflammatory state or the HLA haplotype of the patient, or with polymorphisms in immune genes such as genes encoding tumor-necrosis factor, interleukin-10, or CTLA-4.[7-9] However, the only proven risk factor is the type of mutation in the F8 gene that causes hemophilia A, and more specifically the presence or absence of traces of endogenous FVIII antigen in the circulation of the patient. Indeed, in a mouse model of hemophilia A, FVIII mRNA has been detected in mouse thymus, and intrathymic injection of FVIII into neonatal FVIII knockout mice generates tolerance to subsequent immunization with FVIII.[10,11] These findings strongly suggest that T and B cells reactive to FVIII are deleted through central tolerance mechanisms.

The understanding of the pathophysiological mechanism leading to the development of inhibitors in patients with hemophilia has improved considerably over the last two decades. This process is complex and involves cells, cytokines, and other immune regulatory molecules. This review aims to summarize our current understanding of the molecular mechanisms that lead to inhibitor synthesis and potential areas in need of further investigation.

Primary Immune Response.

Factor endocytosis by APCs and presentation to T-cell. Understanding the location where therapeutic factors encounter the immune system for the first time, the type of antigen presenting cells that are involved in the process and the site where the anti-factor immune response develops is crucial for developing strategies to selectively prevent the onset of the deleterious anti-FVIII and anti-FIX immune response. The first encounter of the infused factor with immune effectors most likely occurs in the spleen. Blood-borne antigens reach the spleen through the splenic artery, which branches either towards the red pulp and interacts with red pulp macrophages or towards the marginal zone of the spleen, which contains three major types of professional APCs: macrophages, B lymphocytes and dendritic cells.[12,13] This view is supported by the work of Navarette et al.[14] where they demonstrated that human FVIII administered to FVIII-deficient mice preferentially accumulates in the marginal zone (MZ) of the spleen. The disruption of splenic germinal centers by intravenous injection of anti-CD154 antibodies also caused a reduction in anti-FVIII antibody titers and abolition of T-cell responses to FVIII.[15] Therefore, identification of the receptors implicated in retention of therapeutic factors in the marginal zone may contribute towards novel strategies aimed at reducing their immunogenicity. In addition, the removal of the spleen or selective in vivo depletion of APCs before repeated FVIII administration reduces the extent of the anti-FVIII immune response.[14] Interestingly, the development of detectable anti-FVIII immune response to therapeutic FVIII was observed in splenectomized animals, indicating that alternative secondary organs, the lymph nodes or possibly the bone marrow, may be involved in the immune response to therapeutic factors as well.[16] On the other hand, another hypothesis is that since bleeding and coagulation create a highly inflammatory microenvironment, therapeutic FVIII/FIX may be captured by antigen-presenting cells at the site of bleeding and then transported to secondary lymphoid organs for presentation to naïve CD4+ T cells. The inflammatory atmosphere could attract locally cells of innate immunity and antigen-presenting cells. The environment may also provide the appropriate signals for the activation of the professional antigen-presenting cells that have endocytosed FVIII and processed FVIII into peptides, about 9-14 amino acids in lengths.[17] FVIII-educated APCs likely migrate to the secondary lymphoid organs which are rich in T-cell like the periarteriolar lymphoid sheath surrounding the splenic artery. There, mature APCs are surveyed by CD4+ T cells that express T cell receptors specific for FVIII peptides bound to MHC class II molecules.

Different types of APCs may be involved in the uptake of therapeutic FVIII in patients. Among these, dendritic cells, macrophages, and B lymphocytes are the most potent. However, the types of APCs differ depending on the "experience" the immune system of the patient has, of exogenous FVIII. In untreated patients who have never been exposed to FVIII, FVIII-specific B lymphocytes have not been triggered and are not likely to be present at a frequency high enough to serve as APCs. B cells and macrophages, although considered professional antigen-presenting cells, most likely do not present FVIII to naıve CD4+ T cells because of the high specificity and strength of immune synapse formation required to activate naıve CD4+ T cells.[18] Therefore, in view of the capacity to stimulate naïve T cells, DCs are likely to be the major APC involved in the primary immune response to clotting factors. DCs are derived from bone marrow and circulate as precursors in blood before entering tissues where they become resident immature DCs that can sense changes in their local environment.[19] Immature

DCs can take up antigen using both receptor- and non-receptor-mediated mechanisms and degrade antigens in endocytic vesicles to produce antigenic peptides capable of binding to MHC-class II.[19] Maturation of DCs requires danger signals provided by exogenous or endogenous stimuli such as pathogen-derived products, inflammatory cytokines, or CD40-CD40 ligand interactions. As DCs mature, they express a high density of MHC-class II molecules complexed with antigenic peptides and upregulate costimulatory molecules. Antigenic peptides complexed with MHC-class II are recognized by the T-cell receptor (TCR) expressed on CD4+ T cells. When human dendritic cells are cultured with FVIII in vitro, this does not lead to DC maturation.[20] The authors concluded that FVIII does not possess inherent danger signals for human DCs. However, certain FVIII products that might have undergone inappropriate production procedures could develop inherent danger signals for the immune system.[21,22] In addition, the monocyte derived DCs used in this study may not be representative of the entire DC population in the body. The causative factors for this difference in the in vitro and in vivo recognition of FVIII by the immune system remains unclear, but, likely, the microenvironment within which FVIII is taken up and presented by immune cells plays an important role in this response.[20,23]

Several endocytic receptors specific for FVIII have been characterized. Members of the low-density lipoprotein receptor (LDLR) family recognize protein structures in the heavy and light chains of FVIII 70}.[24,25] Asialoglycoprotein receptor binds to galactose-ending glycans of the B domain of FVIII.[26] The macrophage mannose receptor (MMR/CD206) interacts with mannose-ending glycans on the A1 and C1 domains of the molecule.[27] Dasgupta et al.[27] used human monocyte-derived dendritic cells to demonstrate that FVIII is endocytosed by the macrophage mannose receptor (CD206) that recognizes mannose-ending glycans on both the heavy and light chains of FVIII. Mechanistically, VWF has been shown to prevent the binding of FVIII to macrophage mannose receptor and block the endocytosis of FVIII by monocyte derived dendritic cells in a dose-dependent manner.[27,28] Therefore, VWF has been proposed to reduce the immunogenicity of FVIII in patients with hemophilia A.[29,30] However, in recent studies, the blockage of the mannose receptors by mannan did not produce the expected effect in reducing uptake by dendritic cells, suggesting that additional, as yet unidentified, endocytic receptors are of clinical significance.[31,32] On the other hand, the monoclonal antibody KM33 targets the FVIII C1 domain, specifically residues Arg2090, Lys2092, and Phe2093.[33,34] It has been shown to completely inhibit FVIII endocytosis by both monocyte-derived dendritic cells and bone marrow-derived dendritic cells by targeting an epitope of FVIII that is essential for its uptake. Specifically, KM33 interferes with the binding of FVIII to low-density lipoprotein receptor–related protein-1 (LRP) and dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) receptors.[32] In vivo administration of KM33 significantly reduced the production of neutralizing antibodies against FVIII.[32] The in vitro and in vivo inhibitory effect of KM33 suggests that these interactive surfaces on the FVIII C1 domain are critical for the initiation of immune response to therapeutic FVIII.

Moreover, infusions of FVIII variant proteins with alanine substitutions at the positions Arg2090, Lys2092, and Phe2093 in FVIII-deficient mice led to reduced T-cell and B-cell responses as compared with wild-type FVIII.[34] Therapeutic monoclonal antibodies to inflammatory cytokines or immunosuppressive agents such as steroids have been shown to limit the activation state and endocytic capacity of APCs.[35,36] Therefore, the inflammatory environment of the patients could be neutralized before or at the time of administration of therapeutic clotting factors. Besides, high-intensity FVIII treatment because of excessive bleeding episodes may allow FVIII to compete more efficiently with other antigens for uptake by APCs, resulting in more efficient presentation of FVIII-derived peptides to CD4+ T cells.[30] As a result, high-intensity FVIII treatment has been linked to higher inhibitor development.[37]

Dendritic cells endocytose and process therapeutic clotting factors into peptides, which are loaded onto the cleft of MHC-II molecules and expressed on the surface of the dendritic cell.[17] During dendritic cell maturation, they also express co-stimulatory molecules such as CD80/86 and CD40 needed for CD4+ T cell activation.[23,38] In the secondary lymphoid organs, mature dendritic cells are surveyed by FVIII-specific CD4+ T cells until cognate MHCII-TCR interactions are established; the engagement of co-stimulatory molecules between the dendritic cell and T cell (i.e., CD40 with CD40L, CD80/CD86 with CD28) occurred; and cytokine secretion by both the dendritic cell and T cell happened to induce T cell activation and proliferation.[39] Several novel strategies have been developed from the understanding of this interactive mechanism. For instance, the abrogation of the cross-talk between APCs and T cells using anti-CD40L monoclonal antibody or CTLA4-Ig constructs showed promising results in FVIII-deficient mice.[15,40] In naïve animals, the use of blocking antibodies to disrupt the cognate interaction between T cells and APCs caused immunological hyporesponsiveness to FVIII, or the partial breakdown of an immune response in FVIII-primed mice.[15,40-42] In humans, only three hemophilia A patients with FVIII inhibitors (> 10 BU/ml) have been treated with anti-CD40L.[43] Inhibitor levels were reported to decrease in these patients. However, more

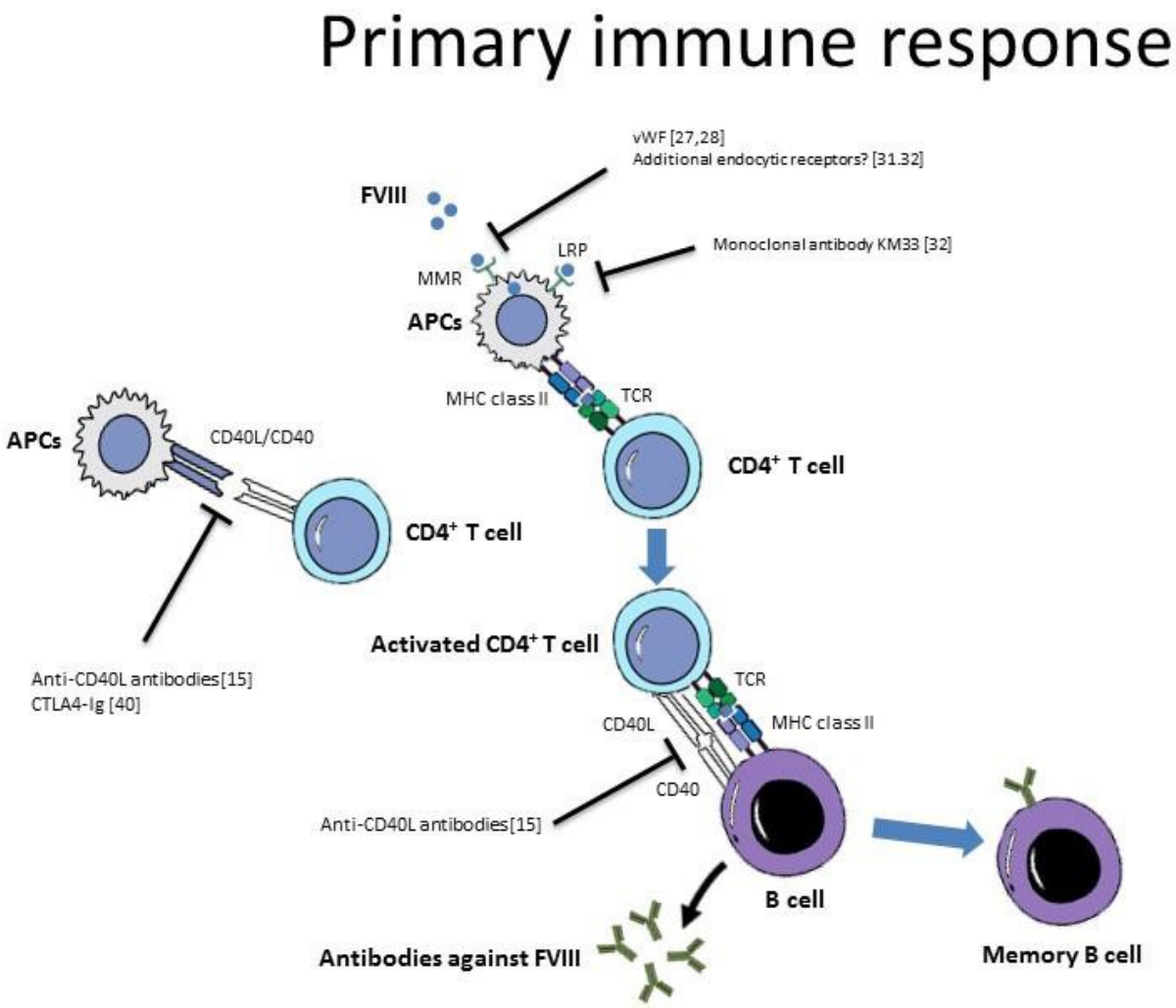

Figure 1. Primary immune response in hemophilia inhibitor development. APC: antigen- presenting cells; MMR: mannose receptor; LRP: lipoprotein receptor-related protein; TCR: T- cell receptor.

evidence suggested that treatment with anti-CD40L was associated with both arterial and venous thromboembolic complications.[44,45] Mechanistically, CD40 and CD40L are both expressed on platelets, and the use of an anti-CD40 antibody can activate platelets, thus increasing the likelihood of thrombotic events. Therefore, CD40-CD40L blockade cannot be considered as a safe alternative for FVIII tolerance induction at the moment.[39]

T-cell presentation to B-cell and B-cell proliferation. Activated CD4+ T cells trafficke to the B cell follicles in the spleen where they activate FVIII specific naïve B cells. Bone marrow derived B cells internalize FVIII via receptor-mediated endocytosis with FVIII-specific membrane-tethered immunoglobulin and interact with activated CD4+ T cells via an MHC II-TCR association.[46] Activated B cells then proliferate and terminally differentiate into FVIII-specific memory B cells or anti-FVIII antibody secreting plasma cells. Memory B cells do not secrete anti-FVIII antibodies. These cells reside in the spleen or bone marrow and quickly terminally differentiate into plasma cells after subsequent exposure to FVIII.[39]

Meanwhile, plasma cells can be either short-lived or, depending on survival factors present during their development, they can reside in the spleen or the bone marrow as long-lived cells.[47,48] In fact, FVIII-specific plasma cells have been demonstrated to survive for a very long time in the absence of further FVIII immunizations in mice.[49] In naïve mice, anti-CD40L blocks the germinal center reaction by preventing cognate T cell-B cell interactions. This would stop the production of new plasma cells and lead to a reduction in the levels of circulating anti-FVIII antibodies in the plasma over time as short-lived plasma cells senesced. However, long-lived plasma cells, which no longer require significant T cell costimulation, could occupy survival niches in the spleen and bone marrow and continue to maintain some level of anti-FVIII Ab production.[39] Strategies to modulate the primary immune response in hemophilia are summarized in **Figure 1**.

Secondary Immune Response. During the secondary immune response, FVIII-specific memory B cells

generated during the primary immune response act as APCs and activate FVIII-specific CD4+ T cells. With the help of CD4+ T cells, FVIII-specific memory B cells further differentiate into ASCs. Meanwhile, uptake of FVIII by other professional APCs, such as the dendritic cells, results in activation of T cells that, in turn, activate new FVIII-specific B cells and thus generate additional ASCs and memory B cells. Several studies investigating the mechanisms of immune tolerance induction demonstrated that high FVIII levels might inhibit memory B cell differentiation.[50,51] Indeed, Reipert et al.[52] discovered that high FVIII concentration could inhibit FVIII-specific memory B cells both in vitro and in vivo. In these studies, splenocytes (depleted of CD138+ plasma cells) were obtained from mice that were repeatedly immunized with FVIII. This CD138- splenocyte pool, therefore, represented a population of memory B cells, which was restimulated in vitro or in vivo, using an adoptive transfer model with increasing concentrations of FVIII. When CD138- splenocytes were restimulated with supraphysiological concentrations of FVIII (between 1 and 20 mcg/mL), potentially mirroring the FVIII levels in some high-dose ITI patients, this memory cell population was incapable of differentiating into anti-FVIII Ab secreting plasma cells. In contrast, physiological FVIII concentrations (0.01–0.1 mcg/mL) supported memory B cell differentiation.

Moreover, Matino et al.[53] demonstrated that induced CD4+FOXP3+ cells were capable of suppressing the differentiation of FVIII-specific memory B cells into FVIII antibody–producing plasma cells in vitro. On the other hand, most antibodies secreted from the plasma cells are mainly of the immunoglobulin IgG1 and IgG4 subtypes and directed against the A2 and/or C2 domains of FVIII. Several epitopes of both neutralizing and non-neutralizing types located outside these, some in the B domain, have also been described.[54,55] The main mechanism by which the antibodies neutralize the factor is by steric hindrance, but the formation of immune complexes and subsequently, the enhanced catabolism as well as hydrolysis have also been suggested.[56] They can interfere with FVIII binding to phospholipids or VWF via binding to the C2 domain.[57,58] Besides, the antibodies can interfere with FVIII binding to FIX or block the intrinsic X-ase activity of the VIIIa-IXa complex.[59,60] Alternatively, the antibodies can increase clearance of VIII via direct proteolysis.[56,61] Regarding non neutralizing antibodies, it remains debated as to whether these antibodies or at least any immune response they provoke, are of clinical significance and should be considered as well.[62-64] Strategies to modulate the secondary immune response in hemophilia are summarized in **Figure 2**.

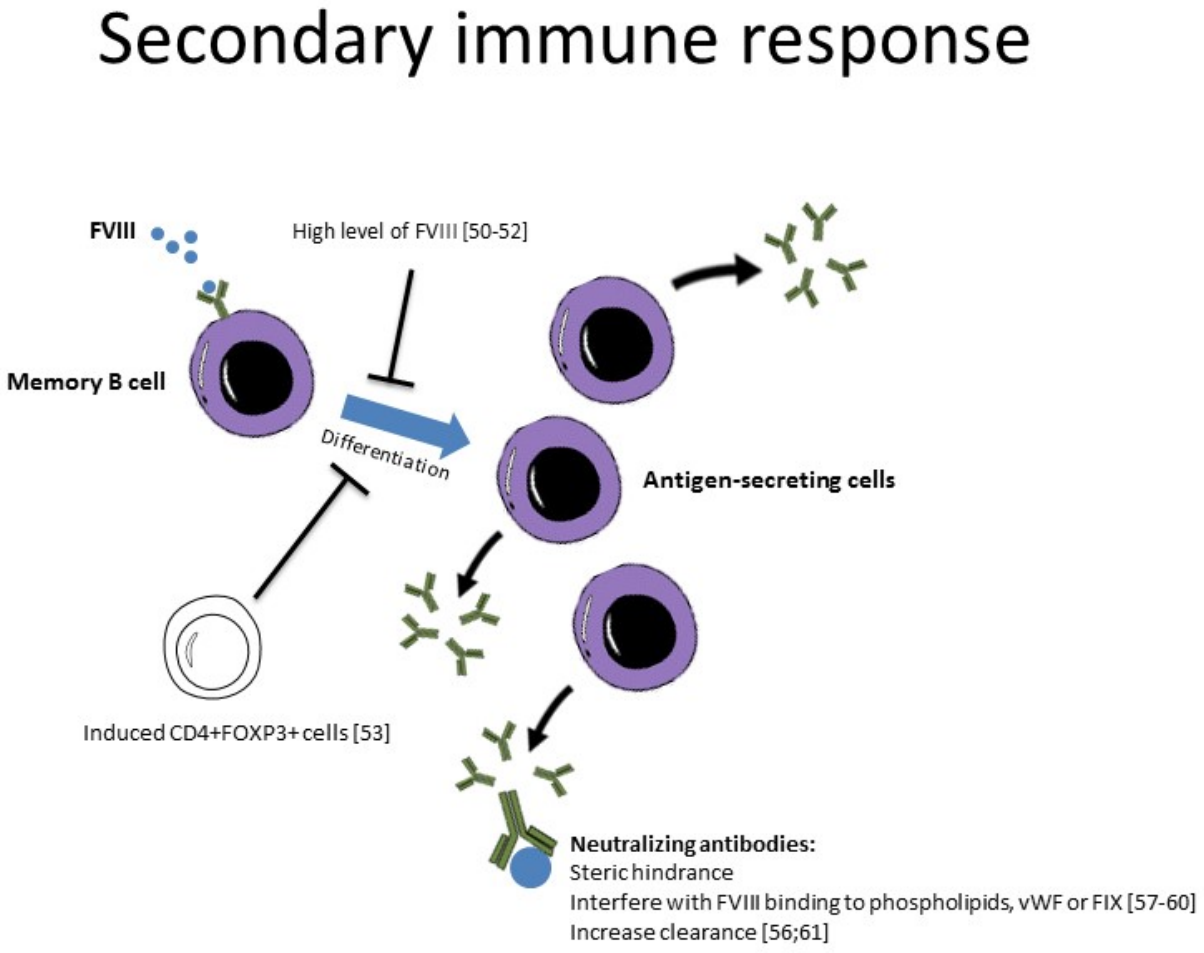

Figure 2. Secondary immune response in haemophilia.

Actors in Inhibitor Development. Inhibitors are high-affinity antibodies. They are primarily immunoglobulin G (IgG) directed against the factor protein.[65] Inhibitors in individuals with acquired hemophilia are often monoclonal. In one study, approximately 80% of individuals with hemophilia A who developed inhibitors had at least two or more independent antibody specificities against factor VIII.[66] There is a distinct spectrum of neutralizing and non-neutralizing antibodies in different cohorts of patients with severe hemophilia A and in healthy individuals.[67] IgG4 and IgG1 were the most abundant IgG subclasses in patients with FVIII inhibitors, while IgG4 was utterly absent in patients without FVIII inhibitors and in healthy subjects.[67] In addition, FVIII-specific antibodies in hemophilia A patients with inhibitors have approximately 100-fold higher apparent affinities than that of antibodies found in patients without inhibitors or in healthy individuals.[65] In patients who are never exposed to the deficient factor, the immune response presumably takes place by dendritic cell pathways, whereas among primed patients with an established immune response, the B cells seem to be the key APCs.[68] The importance of cross-talk between APC and $CD4^+$ T cells has been shown in animal models using antibodies toward costimulatory cell surface molecules interfering with the binding to the CD40 ligand, CD80/86, and CTLA4.[40-42,51,69,70] Indirect evidence of the role that CD4+ cells play in anti-FVIII antibody synthesis comes from the observation that inhibitors may spontaneously disappear in conjunction with an HIV-associated decline in CD4+ counts.[71] More recently, the prevention of inhibitor synthesis in a murine haemophilia model by blockade of costimulatory signals has provided direct evidence that CD4+ cells are indeed essential for the development of an anti-FVIII antibody response.[40] Besides, for the $CD4^+$ T cells to become activated and acquire the capacity to stimulate antigen-specific B-cell differentiation into antibody-secreting plasma cells, additional triggers or alert signals are often required, as suggested in the danger model theory.[72] These danger signals are mainly released by cell death, tissue damage, stress, and systemic inflammatory responses, e.g., interleukins (ILs), heat shock proteins, adenosine triphosphate, reactive oxygen species, and growth factors.[73] Whether a T cell-independent immune response toward FVIII is evoked into producing FVIII-specific antibodies is not completely clear, but this could potentially be of relevance for the formation of non-neutralizing antibodies and/or low-affinity antibodies.[74] Following antigenic stimulation, naive CD4+ cells may differentiate into one of several T-cell subsets that differ in function and cytokine secretion. Th1 cells secrete pro-inflammatory cytokines such as IL-2 and IFN-γ and help in the synthesis of complement-fixing antibodies such as IgG1.[75]

On the other hand, Th2 cells can have a down-regulatory effect on the immune response by secreting anti-inflammatory cytokines such as IL-4 and IL-10, which inhibit the proliferation and function of Th1 cells and antigen-presenting cells. However, Th2 cells can also stimulate B cells that produce certain antibody subclasses such as IgG4. In fact, high-affinity FVIII-specific antibodies found in patients with FVIII inhibitors are predominantly IgG4. This suggests a distinct immune regulatory pathway responsible for the development of FVIII-specific IgG4 associated with FVIII inhibitors.[52,67] Overall, inhibitor production by B cells is controlled by a complex interaction of different CD4+ subsets.[75] Reding et al.[76] demonstrated the importance of both Th1 and Th2 cells in the synthesis of anti-FVIII antibodies. More intense anti-FVIII antibody responses and higher inhibitor titres correlate with a predominance of Th2-driven IgG4. Successful immune tolerance therapy in haemophilia A patients and immunosuppressive therapy in acquired haemophilia patients correlate with a predominance of Th1-driven anti-FVIII antibody.[1]

To further define the role of T cells in the pathogenesis of FVIII inhibitors, Reding and colleagues mapped the CD4+ T-cell epitopes on FVIII.[77,78] They found three immunodominant CD4+ epitopes on the FVIII C2 domain, corresponding to residues 2191–2210, 2241–2290, and 2291–2330.[77] Each of these epitopes overlaps inhibitor-binding sites, suggesting that CD4+ cells recognizing these sequences may be involved in the regulation of inhibitor synthesis. Besides, there is a lack of recognition of specific CD4+ epitopes correlated with inhibitor formation.[77] For instance, the absence of recognition of residues 2191–2210 correlates with inhibitor formation, suggesting that a pathogenic immune response to FVIII results from failure to activate regulatory CD4+ cells specific for certain FVIII sequences. On the other hand, Reding and colleagues found notable differences between the CD4+ epitope repertoires of congenital and acquired haemophilia patients. This suggests different mechanisms of inhibitor formation, which is expected, given that inhibitors are a consequence of an alloimmune response in congenital haemophilia A patients and an autoimmune response in acquired haemophilia patients.

Tregs have also been implicated in the process of inducing tolerance in patients with an established memory using immune tolerance induction therapy. Frequent exposure to the deficient factor in the absence of systemic inflammation may induce Tregs with a subsequent lack of T-helper cells, preventing B-cell differentiation and promoting tolerance through B-cell anergy and/or deletion.[79] High doses in a murine model of hemophilia A irreversibly inhibited the memory B cells via an indirect effect on both APCs and T cells.[50]

The importance of T-regulatory cells in the process of antibody formation has been established, and to date, different subsets of cells with suppressor activities have been defined.[80] Notably, the CD4+CD25+FoxP3+ Treg cells have been well-studied. They originate during thymic T-cell development and are also referred to as natural Tregs.[3] They may also be induced in the periphery from conventional T cells. Treg activation occurs through antigen-specific binding to T-cell receptors, but the suppression appears to be a more nonspecific event, which may add somewhat to the complexity of inhibitor formation. The action of Tregs is multifactorial and includes direct cell contact-dependent mechanisms involving APCs and/or effector T cells, as well as cytokine-mediated suppression of proliferation and differentiation. Tregs may also promote the secretion of suppressive factors by dendritic cells.[81]

Moreover, indoleamine 2,3-dioxygenase 1 (IDO1) is a key regulatory enzyme that supports Treg function and peripheral tolerance in adult life. Matino et al.[53] discovered in both human and hemophilic mouse that defective TLR9-mediated activation of IDO1 induction was associated with an inhibitor-positive status. These findings indicate the novel strategies of improving the IDO1 function in preventing or eradicating inhibitors to therapeutic administered FVIII.[53]

Factor IX Inhibitors. Mechanistic studies on inhibitor development in hemophilia B have been studied extensively compared with hemophilia B. Hemophilia A is four times as frequent as hemophilia B, and the incidence of inhibitors is higher.[1] Further, hemophilia B is often associated with point mutations, which are less commonly associated with inhibitor development, rather than deletions. The extent to which the mechanistic information from hemophilia A can be generalized to hemophilia B is not known and may differ substantially. While the clinical phenotype of haemophilia B is indistinguishable from that of haemophilia A, there are clear differences regarding inhibitor development between the two conditions. The development of FIX inhibitors is much less common than in hemophiliia A, occurring in approximately 5% of those with severe hemophilia B.[82] The majority of those affected (approximately 80%) are high responders, and 50% or more have a history of severe allergic reactions to FIX products.[82] Although the development of pathogenic immune responses against FIX is less common, induction of immune tolerance to FIX is not often successful, occuring in only approximately 15% of treated patients in most series.[82] However, the mechanisms of the immune response to FIX replacement therapy in humans have not been well studied and are thus poorly understood. More work in this area is needed.

Conclusions. The purpose of this review was to summarize the molecular mechanisms of inhibitor development in hemophilia and to identify potential areas in need of further investigation. Understanding the location where therapeutic factors encounter the immune system for the first time, and the site where the anti-factor immune response develops is essential for developing novel strategies towards immune tolerance. Previous work targeting the primary immune response in the splenic germinal centers by anti-CD154 antibodies showed promising results in hemophilia A.[15] Besides the spleen, alternative secondary organs, including the lymph nodes or possibly the bone marrow, may be involved in the immune response to therapeutic factors as well.[16] In view of the capacity to stimulate naïve T cells, dendritic cells are likely to be the major antigen-presenting cells involved in the primary immune response to clotting factors. However, FVIII might not possess inherent danger signals for human dendritic cells. Pfistershammer et al.[20] demonstrated that when human dendritic cells are cultured with FVIII in vitro, this does not lead to DC maturation. The causative factors for this difference in the in vitro and in vivo recognition of FVIII by the immune system remains unclear, but, likely, the microenvironment within which FVIII is taken up and presented by immune cells plays an essential role in this response.[20,23] On the other hand, several endocytic receptors specific for FVIII have been characterized and they can be the potential targets to reduce the immunogenicity of therapeutic factors. For example, VWF has been shown to prevent the binding of FVIII to macrophage mannose receptor and block the endocytosis of FVIII by monocyte derived dendritic cells in a dose-dependent manner.[27,28] In addition, the monoclonal antibody KM33, which targets an epitope of FVIII, has been shown to completely inhibit FVIII endocytosis by dendritic cells. In the secondary lymphoid organs, the engagement of co-stimulatory molecules between the mature dendritic cell and T cell (i.e. CD40 with CD40L, CD80/CD86 with CD28) occurred. A novel treatment using anti-CD40L had been employed in three hemophilia A patients with inhibitors.[43] Although inhibitor levels decreased in these patients, treatment with anti-CD40L was associated with both arterial and venous thromboembolic complications.[44,45] Activated CD4+ T cells trafficke to the B cell follicles in the spleen, where they activate FVIII specific naïve B cells. Activated B cells then proliferate and terminally differentiate into FVIII-specific memory B cells or anti-FVIII antibody secreting plasma cells. Naïve mice treated with anti-CD40L appeared to have the production of new plasma cells stopped, which eventually led to a reduction in the levels of circulating anti-FVIII antibodies in the plasma over time as short-lived plasma cells senesced. During the secondary immune response, FVIII-specific

memory B cells further differentiate into antibody-secreting cells. Antibodies neutralize the therapeutic factor in different ways. They can interfere with FVIII binding to phospholipids or VWF via binding to the C2 domain.[57,58] They can interfere with FVIII binding to FIX or block the intrinsic X-ase activity of the VIIIa-IXa complex.[59,60] Alternatively, the antibodies can increase clearance of VIII via direct proteolysis.[56,61] Several studies investigating the mechanisms of immune tolerance induction demonstrated that high FVIII levels might inhibit memory B cell differentiation.[50,51]

Regarding nonneutralizing antibodies, it remains debated as to whether these antibodies, or at least any immune response they provoke, are of clinical significance and should be considered as well.[62-64] In addition, high-affinity FVIII-specific antibodies found in patients with FVIII inhibitors are predominantly IgG4, and that suggests a distinct immune regulatory pathway responsible for the development of FVIII-specific IgG4 associated with FVIII inhibitors.[52,67] Overall, the prevention of antibody development against FVIII during replacement therapy of patients with hemophilia A remains a major goal in the design of future treatment strategies. Identification of early biomarkers that predict inhibitor development in previously untreated patients with hemophilia A will assist in risk identification and possible early intervention strategies. In the last decade, advances have been made in our understanding of the mechanism of the immune response to therapeutic factors in hemophilia patients. A clear understanding of the relevance of these mechanisms in the context of successful immune tolerance therapy, and ultimately gene therapy, awaits further study.

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