General introduction
**Hemophilia A**

Hemophilia A is hereditary X-linked disorder caused by dysfunction or absence of blood coagulation factor VIII. The bleeding syndrome has been recognized as early as in the fifth century, but only since 1937 was linked to a deficiency of a plasma component, named in 1962 “factor VIII”. \(^1\) Depending on the severity of the disease, it can be divided into three categories: mild (5-25% of normal plasma levels of FVIII), moderate (1-5%) and severe (<1%) hemophilia A. In its severe form it can lead to spontaneous joint and muscle bleeds, which consequently can cause deformation of joints requiring surgical intervention. Trauma can cause life-threatening internal bleedings and hemorrhages. Treating hemophilia became possible in 1964, when Judith Graham Pool, working at Stanford University, described how to obtain cryoprecipitate from human plasma. \(^2\) Since then, so-called replacement therapy comprising regular injections of FVIII of either recombinant or plasma-derived origin became commonly used in hemophilia care. However, such treatment is hampered by high costs of the FVIII products – it is estimated that more than 75% of the hemophilia community worldwide receive either inadequate or no treatment whatsoever. \(^3\)

In response to frequent FVIII infusions, a subset of patients develops anti-FVIII antibodies. In severe hemophilia A, inhibitors develop after a median of 10 to 15 days of treatment with FVIII. \(^4\) After 50 exposure days, the cumulative incidence of inhibitors reaches a plateau, after which the occurrence of inhibitors is exceptional. \(^5\) Anti-FVIII antibodies can rapidly inhibit FVIII function, rendering FVIII infusion therapy ineffective. Inhibitory antibodies interfere with binding of FVIII to other coagulation factors such as factor IIa, IXa and X or to phospholipids. \(^6,7\) Non-inhibitory antibodies can also compromise hemophilia treatment by influencing FVIII stability and/or its pharmacokinetics by interfering with binding to von Willebrand factor (VWF). \(^8,9\) The majority of inhibitory antibodies directed towards FVIII are of subclass IgG1 and IgG4. \(^10-12\)

**Factor VIII: structure and function**

FVIII is synthesized as a polypeptide chain comprising a signal peptide of 19 amino acids and a mature protein of 2332 residues. FVIII consists of three A, two C domains and one unique B domain that are arranged in the following order: A1-a1-A2-a2-B-a3-A3-C1-C2 (Figure 1). \(^13,14\) The a1 (residues 337-372), a2 (residues 711-740) and a3 (residues 1649-1689) regions bordering the A domains are enriched for aspartic and glutamic acid residues. \(^13\) In plasma, FVIII circulates as a hetero-dimer consisting of a 90-220 kDa heavy chain that is non-covalently linked to a 80 kDa light chain (Figure 1). \(^15\) Cleavage at Arg1648 by as yet unidentified protease releases the light chain (a3-A3-C1-C2); additional processing at various positions (including Arg740 and Arg1312) within the B domain yields a heavy chain (A1-a1-A2-a2-B) that is heterogeneous in size. In the circulation, FVIII is tightly associated with its carrier protein – von Willebrand factor (VWF), which protects it from proteolytic degradation and premature clearance. \(^16-20\) Sulfation of Tyr1680 within the acidic a3 region is required for high affinity binding of FVIII to VWF. \(^21-23\) Also residues in the C1 and C2 domain have been implicated...
in binding of FVIII to VWF.\textsuperscript{16,21-25} FVIII circulates as an inactive precursor that can only act as a cofactor for the FIXa-dependent conversion of FX following its activation by thrombin or FXa.\textsuperscript{26} Cleavage by thrombin at Arg1689 releases the acidic α3 region which promotes rapid disassembly of the FVIII-VWF complex\textsuperscript{27,28}, cleavage at Arg372 and Arg740 results in a hetero-trimeric molecule comprising the A3-C1-C2, A1-α1 and A2-α2 domains.\textsuperscript{27} The resulting hetero-trimer can efficiently catalyze the conversion of factor X to Xa by factor IXa on phospholipid surfaces.\textsuperscript{14} Dissociation of the A2 domain from hetero-trimeric FVIII results in a rapid decline of FVIII cofactor activity.\textsuperscript{29-31} Also cleavage of FVIII by activated protein C at positions Arg336 and Arg562 abolishes cofactor activity of activated FVIII.\textsuperscript{32,33} Residues 1811-1818 in the A3 domain and residues 558-565 in the A2 domain have been shown to contribute to the binding of factor IXa.\textsuperscript{34-37} Limited information is available with respect to the binding site for factor X on activated FVIII; binding sites for factor X in the C2 domain and the α1 region have been proposed.\textsuperscript{38,39} It is now well-established that both the C1 and C2 domain contribute to binding of FVIII to negatively charged phospholipids.\textsuperscript{40-44} Analysis of the three-dimensional structure of the C2 domain and site-directed mutagenesis have firmly implicated a role for Met2199, Phe2200, Leu2251 and Leu2252 in the binding of FVIII to negatively charged phospholipids.\textsuperscript{45-48} More recent studies have identified an exposed surface loop in the C1 domain harbouring Arg2090, Gln2091, Lys2092 and Phe2093 that modulates binding of FVIII to surfaces containing a low percentage of negatively charged, phosphatidylserine-containing phospholipids.\textsuperscript{42,43} Figure 1 provides an overview of interactive sites on FVIII for its major binding partners.

**Figure 1. Structure of blood coagulation factor VIII (FVIII).** Upper left panel shows schematic domain organization of FVIII; lower left panel – schematic representation of circulating FVIII, where the heavy (A1-A2-B) and the light (A3-C1-C2) chain are non-covalently linked via a metal ion-dependent interaction between the A1 and A3 domain. In activated FVIII, the A1 and A3 domains remain non-covalently bound, while the A2 domain is weakly associated with the A1/A3-C1-C2 dimer. Right panel provides a crystal structure of B domain-deleted FVIII (pdb code 3cdz). Interactive sites for phospholipids, VWF, FIXa and FX are indicated in the model.
Epitope mapping studies have revealed that inhibitory antibodies that develop in patients with hemophilia A bind to the A2 and C2 domain of FVIII. More detailed binding studies have shown that residues 484-508 provide a major binding site for anti-A2 domain antibodies. The mode of action of anti-A2 inhibitors is not yet entirely clear; an early study by Lollar and co-workers suggested that anti-A2 antibodies act as non-competitive inhibitors of intrinsic Factor X activation complex by blocking the conversion of FXase/FX complex to the transition state. Follow-up studies using isolated A2 domain suggested that anti-A2 antibodies directly inhibit the interaction of A2 subunit with factor IXa, thus abrogating the contribution of this subunit to cofactor activity. Anti-C2 domain antibodies have been shown to bind to exposed residues overlapping with the phospholipid binding site. Co-crystalization of a human monoclonal anti-C2 domain antibody, BO2C11, revealed that Arg2215, Arg2220, Met2199, Phe2200, Val2223, Leu2251 and Leu2252 comprise contact residues for this inhibitor. Inhibitory antibodies directed towards residues 1811-1818 in the A3 domain have also been detected in patients with hemophilia A. Antibodies binding to these sites have been shown to limit the binding of FIXa to FVIII. Moreover, in a small number of patients antibodies directed towards the acidic a1-region have been identified.

Replacement therapy and inhibitor development in hemophilia A
The development of inhibitors occurs in approximately 5% of mild or moderate hemophilia A patients, and in 25% of severe hemophilia A patients. The low prevalence of inhibitor development in patients with mild and moderate hemophilia A is most likely caused by the presence of endogenous levels of circulating FVIII which render this group of patients tolerant to subsequent replacement therapy. The lack of endogenous levels of circulating FVIII most likely underlies the increased frequency of FVIII inhibitors in patients with severe hemophilia A. A large number of studies have addressed the correlation between FVIII genotype and inhibitor development. Patients carrying intron 22 inversions, nonsense mutations or large deletions are more prone to develop inhibitors than those with small deletions and missense mutations. Nonsense mutations that affect the light chain of FVIII are more frequently associated with inhibitor development than those present in the FVIII heavy chain. Formation of inhibitors in mild hemophilia associates with a limited number of high-risk FVIII mutations within the A2, C1 or C2 domain, namely Arg593Cys, Arg2150His or Trp2229Cys. Why only a fraction of patients generate antibodies against FVIII is still poorly understood. Both treatment-related and genetic risk factors have been shown to contribute to inhibitor development in hemophilia A. Intensity of FVIII treatment is an acknowledged risk factor, as FVIII administered to treat bleeding episodes or to support surgery delivers high concentrations of FVIII that could promote antibody development. Apart from FVIII gene mutations, polymorphisms within the IL-10 and TNFA gene have been associated with inhibitor development. Interestingly, a C/T polymorphism in the promoter region of the CTLA-4 gene was overrepresented in hemophilia A patients without inhibitors suggesting a protective role of this SNP in antibody
formation. Several studies have explored whether inhibitor development is linked to type of FVIII product. There are conflicting findings about the immunogenicity of recombinant versus plasma derived FVIII. Results from a large multicenter cohort study suggest that the risk of inhibitor formation is similar for recombinant and plasma derived FVIII products. Also the von Willebrand factor content of therapeutic FVIII products seems not to be associated with the risk of inhibitor development.

**FVIII and the immune system**

It is now well-established that the formation of high-affinity IgG molecules requires FVIII-specific CD4+ T-cell help. Initial evidence for involvement of T cells in the development of inhibitors in hemophilia A patients came from studies involving HIV-positive individuals, that due to diminished number of CD4+ T cells showed also decline in anti-FVIII antibody responses. The first step leading to activation of specific T- and B-cell responses is recognition of antigen by specialized antigen-presenting cells (APCs). Subsequently, antigen is processed into small peptides which are loaded on MHC class II. Presentation of antigen-derived peptides on the surface of APCs triggers the activation of antigen-specific CD4+ T cells (Figure 2). In the past years, several studies have addressed how FVIII is processed by APCs. Although a role for several receptors has been suggested (for a review see Chapter 2), the exact mechanism of FVIII endocytosis by APCs remains unclear. APCs, equipped with receptors recognizing foreign, pathogen-derived molecules (so-called pathogen-recognition receptors, PRRs), are able to sense “danger”, which prompts maturation of these cells. Upon maturation, they upregulate a number of co-stimulatory molecules such as CD40, CTLA-4, CD80 and CD86 and due to their simultaneous ability to release cytokines APCs can activate and modulate antigen-specific T cell responses (Figure 2). The importance of co-stimulatory mechanism for FVIII-specific T-cell activation has been illustrated by several studies on the blockade of CD40/CD40L interactions in vivo. CD40/CD40L ligation provides a key event to induce humoral responses against antigens; furthermore, blockage of CD40/CD40L interaction leads to long-lasting tolerance in mice. However, even though the disruption of CD40/CD40L interaction by pre-administration of a monoclonal antibody targeting CD40L resulted in deficient immune responses against FVIII in vivo, it failed to induce long-lasting tolerance. Clinical trials suggested that administration of a humanized anti-CD40 ligand antibody (hu5c8) can block anamnestic responses to factor VIII; however it remained unclear whether that effect would persist and result in long-lasting tolerance. Moreover, due to tromboembolic complications in patients treated with hu5c8, this approach for treatment has been discontinued.

During TCR activation in a particular cytokine milieu, naive CD4+ T cells may differentiate into one of several lineages of T helper (Th) cells including Th1, Th2, Th17 and regulatory T cells (Tregs), as defined by their pattern of cytokine production and function. Generally, Th1 cells promote cellular immunity; Th2 cells mediate humoral immunity; Th17 cells play an important role in clearing pathogens during
host defense reactions and in inducing tissue inflammation in autoimmune disease\textsuperscript{94}; Tregs are capable of inducing tolerance by suppressing T- and B-cell responses. T-cell responses against FVIII are of a polyclonal origin and directed against multiple epitopes present in different domains of FVIII.\textsuperscript{95} Th1\textsuperscript{10}, Th2\textsuperscript{10} as well as Th17\textsuperscript{96} cells have been shown to contribute to FVIII-specific immune responses in hemophilia subjects. Eventually, FVIII-specific T cells are able to activate FVIII-specific B cells and subsequently induce affinity maturation and class-switching of immunoglobulin genes. As a consequence, FVIII-specific long-living plasma cells and memory B cells are generated, which are able to rapidly respond to re-exposure to FVIII.\textsuperscript{97,98} FVIII-specific B cell responses are also, similar to T-cell responses, of a polyclonal origin and directed against various epitopes. Determination of classes and subclasses of anti-FVIII antibodies revealed dominant contribution of IgGs and their subclasses – IgG1, IgG2 and IgG4.\textsuperscript{8,10,12} Generated antibodies can inhibit FVIII function by interfering with its interaction with other coagulation factors (mainly A2 and A3-C1 antibodies) and/or phospholipids (C1/C2-directed antibodies), as described earlier. It has been reported, that a subset of anti-factor VIII IgG hydrolyzes FVIII. These so-called catalytic antibodies were found in over 50% of inhibitor-positive patients with severe hemophilia A, but not in inhibitor-negative individuals.\textsuperscript{100} FVIII-hydrolyzing IgG from each patient tested exhibit multiple cleavage sites on FVIII and the specificity of cleavage varied from one patient to another.\textsuperscript{100} Circulating antibodies that do not inhibit FVIII function can influence FVIII half-life, either by interfering with binding of FVIII to VWF or by formation of immune complexes that can be efficiently cleared via Fc receptors.

\begin{figure}
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\caption{Simplified overview of the development of humoral immune responses. Antigen is endocytosed by antigen-presenting cell (such as dendritic cell), processed and presented on MHC class II. For efficient activation, co-stimulatory molecules present on APCs and T cell receptors need to interact, while cytokines released by APCs determine the future direction of ongoing T-cell responses. Once primed, T cells can activate B cells in an antigen-specific manner, which leads to formation of long-living plasma cells producing high-affinity antibodies.}
\end{figure}
Immune tolerance induction (ITI) therapy – eradication of inhibitors
Development of inhibitors is a serious complication in hemophilia care. The magnitude of antibody responses is quantified by a functional assay and expressed in Bethesda units (BU). Patients with low titer of inhibitors (< 5 BU/ml) can be treated with higher and/or more frequent doses of FVIII, which leads to saturation of pre-existing inhibitors and still provides enough FVIII to restore hemostasis and normal coagulation. For patients with higher inhibitor titers (>5 BU/ml), simple infusion therapy becomes ineffective, therefore bypassing agents such as activated factor VII or activated prothrombin-complex concentrates need to be used.\textsuperscript{101,102} Simultaneously, eradication of inhibitors and immune tolerance induction (ITI) therapy is introduced. ITI comprises regular injections of FVIII for a period varying from several weeks up to two years and since its first description in 1977 \textsuperscript{103} remains the only strategy that proved to both eradicate FVIII inhibitors as well as lead to induction of FVIII-specific immune tolerance. The most commonly used protocols, known as the Bonn \textsuperscript{104}, the Van Creveld \textsuperscript{105} and the Malmö \textsuperscript{106–108} protocols, although considerably different \textsuperscript{65}, result in comparable success rates (up to 87%). A recent study showed that high-dose ITI leads to faster recovery and tolerance induction, accompanied by fewer bleeding episodes as compared to low-dose ITI.\textsuperscript{109} Van Helden and co-workers showed a correlation between distribution of IgG subclasses of anti-FVIII antibodies and outcome of ITI therapy. A predominance of IgG4 antibodies was observed in patients who needed prolonged ITI treatment.\textsuperscript{11} Also complications, such as venous catheter infection, can prolong the course of ITI or even lead to its failure.\textsuperscript{110} The immunological mechanisms underlying success of ITI therapy remain unclear. In a naïve, non-primed environment, chronic exposure to high doses of antigen would activate regulatory T cells able to suppress antigen-specific (in this case FVIII-specific) effector T cells, resulting in tolerance induction.\textsuperscript{111,112} Consequently, no T cell help would be provided to FVIII-specific B cells that could not differentiate into long-living antibody producing plasma cells and, as a result, would be depleted. This scenario is however unlikely to happen in patients with pre-existing antibodies that have enriched populations of FVIII-specific memory T and B cells. In this case, memory B cells serve as highly efficient antigen-presenting cells, able to effectively re-stimulate FVIII-specific memory T cells. Moreover, upon encounter with antigen, memory B cells rapidly differentiate into specific antibody producing plasma cells, enriching already pre-existing pool of such cells. However, the amount of antigen seems to be crucial factor for an optimal stimulation of memory B cells.\textsuperscript{113} Studies using FVIII-deficient mice showed that concentrations of FVIII below the physiologic plasma concentration of 0.1 μg/ml (1 U/mL) restimulate FVIII-specific memory B cells and induce their differentiation into antibody-secreting cells (ASCs).\textsuperscript{113} However, high concentrations (above 0.1 μg/ml), inhibit memory B cell restimulation and prevent the formation of ASCs.\textsuperscript{113} The inhibition of FVIII-specific memory B-cell responses seems to be irreversible and not mediated by FVIII-specific T cells. Such depletion of memory B-cell can be an early event in the inhibitors eradication in patients undergoing ITI therapy, who receive high doses of FVIII. Gilles and
colleagues followed two patients with FVIII inhibitors during their course of ITI and suggested that induction of anti-idiotypic antibodies, neutralizing the existing anti-FVIII antibodies, might be one of the reasons underlying success of ITI.

Animal models of hemophilia A
In 1995 Bi and co-workers introduced two mouse models for hemophilia A, which over the years proved to be very useful for broadening our knowledge on induction and modulation of immune responses against FVIII. Targeted disruption of exon 16 (E16-KO mice) or exon 17 (E17-KO) resulted in the absence of functional FVIII in the circulation (<1% normal FVIII activity). Both strains displayed a bleeding tendency, which could be corrected by infusions with human FVIII. Similar to hemophilia A patients, FVIII-deficient mice develop an immune response following repetitive intravenous injections of therapeutic doses of FVIII. Generated antibodies are directed both to the light as well as to the heavy chain of FVIII, persist in the circulation for a long time and their development is strictly dependent on CD4+ T helper cells. Cytokine profiles of factor VIII-specific T cells indicate that the regulation of the anti-FVIII antibody response in hemophilic mice involves both Th1- and Th2-type cells. Over the years, studies involving FVIII-deficient mice enabled us to gain more knowledge regarding modulation of immune response to FVIII and tolerance induction. Furthermore, it allowed for evaluation of a number of novel therapeutic approaches to prevent or eradicate inhibitor development in hemophilia A. Administration of complexes of FVIII and phosphatidylserine (PS) liposomes resulted in reduced antibody formation against FVIII in hemophilia A mice. PS associated with apoptotic cells is known to induce anti-inflammatory responses in APCs, moreover, PS liposomes reduce the maturation, pro-inflammatory cytokine production and T cell priming of APCs. Formation of complex between FVIII and phospholipid molecules is mediated by residues located in the C1 and C2 domains of FVIII. Therefore, a possible alternative explanation of the observed inhibitory effect is that PS occupies residues of FVIII that are crucial for its endocytosis by APCs. CTLA4-IgG, blocking the co-stimulatory interaction between B7 and CD28, has been shown to transiently inhibit anti-FVIII antibody formation in hemophilia A mice. An independent study revealed that simultaneous blockage of CD40-CD40L and B7-CD28 pathways abolishes development of inhibitors and promotes long-term immune tolerance specific for FVIII. CD3 antibody has been studied as a tolerance-inducing agent for several autoimmune and inflammatory diseases. It modulates the CD3–T-cell receptor (TCR) complex and leads to anergy or apoptosis of effector T cells, or to the expansion of regulatory CD4+CD25+ T cells. Consequently, anti-CD3 treatment proved to successfully prevent inhibitor formation in hemophilia A mice with both BALB/c and C57BL/6 background. Rituximab, a therapeutic anti-CD20 antibody, has been used for several years to treat patients with inhibitors, although with various success rates. In hemophilia A mice, depletion of B cells using anti-CD20 treatment prevented increase in inhibitor
titers in FVIII-challenged animals. However, hyporesponsiveness to FVIII was sustained over time only when an anti-CD20 antibody of subclass IgG1 was used, which, in contrast to the other isotype tested – IgG2a, did not deplete marginal zone B cells. Moreover, treatment with anti-CD20 IgG1, but not IgG2a, was accompanied by substantial increase of splenic regulatory T cells, implicating a possible tolerogenic role for the remaining B cell population. A recent study by Sack and colleagues, where anti-CD20 treatment was combined with AAV-based gene therapy, showed that B cell depletion can render FVIII-deficient mice hyporesponsive to FVIII, but results were dependent on the genetic background of the strain used. Transduction with retroviral constructs encoding FVIII A2- or C2-domain in frame with an IgG heavy chain backbone of B cell blasts has been shown to induce immune tolerance to FVIII in hemophilic mice. Mice treated with transduced B cells showed reduced inhibitor titers, which were persistently low after additional challenges with FVIII. Furthermore, the lower antibody titers correlated with an increased frequency of FVIII-specific regulatory T cells. Factor VIII deficient mice have also been successfully used to study the presence and persistence of memory B cells, as in detailed described in the previous paragraph. Bril and colleagues generated transgenic mice expressing human FVIII with the Arg593 to Cys mutation, which is associated with mild hemophilia phenotype. Unlike E16-KO or E17-KO mice, these animals did not develop inhibitor titers upon repetitive intravenous injections of human FVIII, however induction of an immune response took place after subcutaneous FVIII administration in the presence of a strong adjuvant. A similar model was described by van Helden and colleagues who constructed a transgenic mouse expressing full length human FVIII. In accordance with findings by Bril and co-workers no inhibitor formation was observed in this model following the intravenous administration of human FVIII. Interestingly, infusion of PEGylated FVIII evoked an immune response in transgenic mice expressing human FVIII. These results suggest that transgenic mouse models that express human FVIII are useful models for assessing the potential immunogenicity of genetically or chemically modified FVIII variants. More recently, the development of a humanized hemophilic E17 HLA-DRB1*1501 mouse model has been described. Humanized mice have been utilized to study the regulation of HLA class II-restricted immune responses to various antigens and they proved to be highly suitable for in vivo research into the mechanistic basis of human diseases associated with activation of CD4+ T cells. HLA-DRB1*1501 was selected due to a strong connection between this haplotype and many immunologic diseases, as well as a previously noted link between inhibitor incidence and DRB1*1501 in patients with severe hemophilia A. Despite some obvious limitations, such new models can be used to analyze the differences in FVIII-specific T-cell repertoire between mice and men.
Scope of the thesis

Understanding the recognition of FVIII by antigen-presenting cells and subsequent activation of specific T and B cells is essential for development of new strategies for treatment and/or prevention of inhibitor formation to FVIII in hemophilia A patients. In this thesis we explored the mechanism of FVIII endocytosis by both human and mouse dendritic cells (Chapter 3 and Chapter 6). A search for structural determinants important for immune recognition of FVIII revealed that C1 domain-targeting monoclonal antibody KM33 prevents the uptake of FVIII by APCs and delays the formation of anti-FVIII antibodies in hemophilia A mice. Chapter 4 follows up on this finding and shows that modification of C1 domain residues crucial for KM33 binding diminishes FVIII uptake by dendritic cells. Upon in vivo administration in FVIII-deficient mice these C1 domain variants developed significantly lower anti-FVIII antibody titers and reduced CD4^+ T cell responses. In Chapter 5 we address the role of immune complex formation in FVIII endocytosis by APCs and its influence on subsequent FVIII-specific T cell responses. Chapter 6 elaborates in more detail on the possible mechanism of FVIII endocytosis by human dendritic cells and the potential role of the C2 domain in this process. Together our findings provide more insight into immune recognition of FVIII that can be utilized to develop novel strategies for treatment or prevention of inhibitor formation in hemophilia A.
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