Molecular determinants of FVIII immunogenicity in hemophilia A
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General discussion
Formation of anti-drug antibodies is a major challenge in clinical care. Treatment of hemophilia A, comprising regular intravenous injections of either plasma-derived or recombinant FVIII, is often compromised by the development of an immune response to FVIII. The intrinsic immunogenicity of FVIII gives rise to antibodies (so-called inhibitors) that limit the pro-coagulant activity of FVIII. The risk for inhibitor development is associated with both genetic and treatment-related factors. One of them is disease severity – in patients with severe hemophilia A the risk of inhibitor formation is about 5-fold higher when compared to that in patients with mild or moderate hemophilia A. Due to a lack of clonal deletion of antigen-specific T cells in the thymus FVIII is recognized as a foreign protein by the immune system in patients with severe hemophilia A. In patients with mild hemophilia A the presence of endogenous FVIII eliminates FVIII-specific T cells during ontogeny of the immune system. Therefore antibody formation rarely occurs in mild and moderate patients; however, if present, it is mostly associated with missense mutations in the A2, C1 or C2 domains. Analysis of mild hemophilia A patients with mutations at positions Arg593, Arg2150 and Ala2201 revealed that CD4+ T cells recognizing peptide-sequences overlapping Arg593, Arg2150 or Ala2201 are present in these patients. Lack of elimination of CD4+ T cells targeting peptides containing these residues most likely underlies the formation of inhibitory antibodies in a subset of patients with mild hemophilia A. Recently, a new hemophilia-related mild mutation, Pro1809Leu (A3 domain), has been described. Interestingly, a patient carrying this mutation developed inhibitors directed not to the A3 domain, but the C2 domain, suggesting that this novel mutation may actually alter the conformation of FVIII molecule, resulting in enhanced immunogenicity of the C2 domain.

Formation of high affinity anti-FVIII is a CD4+ T-cell dependent process. In this thesis we focused on endocytosis of FVIII by antigen-presenting cells, which represents the initial step in development of immune responses, which determines further fate of antigen-specific T- and B-cells. Chapter 3 provides insights into different receptors present on dendritic cells and their role in uptake of FVIII. Moreover, we show that the C1 domain contains a major determinant for immune recognition of FVIII by APCs. Chapter 4 follows up on these findings – we demonstrate that modification of an exposed loop in the C1 domain reduced endocytosis of FVIII by human and murine APCs. Administration of this novel C1 domain variant in vivo leads to diminished FVIII-specific T- and B-cell responses. In Chapter 6 we come back to the original research question concerning mechanism of FVIII endocytosis by dendritic cells, characterizing in more details intracellular events accompanying its uptake. In chapter 5 we studied the effect of immune complex formation on the endocytosis of FVIII. Here, results from each chapter are discussed in the light of current knowledge and studies performed by other investigators.

In search for receptor mediating endocytosis of FVIII by dendritic cells
The mechanism of FVIII uptake by antigen-presenting cells and its immune recognition has been a matter of debate over recent years. In chapters
3, 4 and 6 we focused on unraveling the mechanism of FVIII endocytosis by dendritic cells. We show that uptake of FVIII is dependent on bivalent ion-dependent receptors, as it can be blocked by the presence of EDTA. It has been demonstrated that FVIII can bind in vitro to endocytic receptors such as mannose receptor (MR), DC-SIGN, LRP and Siglec-5 (Figure 1). The presence of mannose-terminated glycans at Asn239 (A1 domain) and Asn2118 (C1 domain), the second most prevalent type of glycans on FVIII, would suggest that mannan-sensitive C-type lectins such as MR and DC-SIGN could play a role in FVIII endocytosis. Dasgupta and colleagues have shown that mannan can indeed block both FVIII endocytosis by human dendritic cells as well as subsequent presentation to and proliferation of FVIII-specific T-cell clone. However, our results (see Chapter 3) revealed that siRNA-mediated knock down of both MR as well as DC-SIGN did not influence FVIII endocytosis, even when both receptors were targeted simultaneously. Moreover, C1 domain-targeting antibody KM33, which abrogates uptake of FVIII by DCs, did not interfere with binding of FVIII to MR. Altogether, these data suggest that neither MR nor DC-SIGN are essential for FVIII endocytosis by dendritic cells. LDL receptor related protein (LRP) has been shown to serve as a major clearance receptor for FVIII. However, its role in FVIII endocytosis by dendritic cells has been excluded by others, which was further supported also by our findings. Only recently, Siglec-5 has been proposed as a novel interacting partner for FVIII. This member of sialic acid-binding immunoglobulin-like lectins is expressed on cells with hematopoietic

![Figure 1. Receptors implicated in FVIII endocytosis. Schematic overview of endocytic receptors implicated in endocytosis of FVIII. From the left to the right: mannose receptor (MR), dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), low density lipoprotein receptor-related protein (LRP) and sialic acid-binding immunoglobulin-like lectin 5 (Siglec-5). EGF – epidermal growth factor.](image-url)
origin, such as granulocytes, monocytes/macrophages, B cells as well as subset of activated dendritic cells. The presence of Siglec-5 on macrophages may suggest its possible involvement in FVIII clearance; however, lack of expression on immature dendritic cells questions its contribution to induction of FVIII-specific immune responses. FVIII circulates in plasma as a glycoprotein complexed with its chaperone molecule, von Willebrand factor (VWF). VWF protects FVIII from premature activation, rapid clearance and degradation by circulating proteases. In vitro, VWF has been shown to block endocytosis of FVIII by dendritic cells. It proved to have similar effect on macrophages, however those cells seems to start to internalize FVIII-VWF complex once shear stress conditions are applied. It has been suggested that the local shear stress environment in the liver and spleen may be sufficient to trigger such events. Nonetheless, full physiological relevance for these studies remains to be established.

Role for the C1 domain in induction of immune responses to FVIII
While the question regarding the nature of receptor involved in FVIII endocytosis has not yet been definitively answered, the molecular determinant on FVIII that is responsible for its immune recognition seems closer to being unraveled. Recombinant monoclonal antibody KM33, directed to the C1 domain of FVIII was able to efficiently block its endocytosis by dendritic cells. Moreover, in vivo administration of KM33 led to delay of FVIII-specific immune responses (Chapter 3). Modification of KM33 epitope by introducing alanine substitutions in positions 2090, 2092 and 2093, resulted in molecule with impaired ability to enter antigen-presenting cells (Chapter 4). This FVIII mutein proved to be less immunogenic also in vivo, as repeated infusions in hemophilia mice led to diminished T- and B-cell responses as compared to wild-type FVIII.

Both C1 and C2 domain were shown to play major role in membrane binding of FVIII. While the phospholipid binding properties of the C2 domain have been characterized several years ago, a role for the C1 domain has been suggested only recently. The C1 domain is thought to exist in locked orientation, determined by multiple binding sites with the A3 domain; the C2 domain, on the other hand, seems to be more flexible, which changes upon membrane binding. It has been suggested that C1 domain is the first one that binds to the lipid layer, thereby locking C2 domain in conformation which allows its high affinity binding to the membrane. The important role for the C1 domain in securing the optimal conformation and position of the C2 domain, made us wonder whether the C2 domain also contribute to FVIII endocytosis by dendritic cells. However, as shown in chapter 6, its role seems to be only marginal.

Recently, a novel mild-hemophilia associated mutation – Pro1809Leu was described. A patient bearing that mutation still required additional infusions of recombinant or plasma-derived products to restore normal FVIII activity, and, as a result of the therapy, he developed an inhibitor (>5 BU/ml). It turned out that the inhibitors were active only against exogenous proteins, but not the patient’s own, endogenous FVIII. However, the epitope was located in the C2, not A3 domain, which was carrying the original mutation. This suggests
that modification of the interaction surface between A3 and C1 domain can influence the conformation of the C2 domain, resulting in this case in exposure of neo-epitopes, a change in the immunogenicity of the C2 domain and, as a consequence – inhibitor development.

Structural determinants involved in FVIII endocytosis: clearance vs. immunogenicity
Despite numerous differences between mechanism of FVIII clearance and its immune recognition, with a role for LRP being clearly distinct for both processes, a central role for the C1 domain seems to be a common denominator. KM33 antibody blocks both LRP-mediated FVIII uptake, as well as endocytosis by dendritic cells and macrophages. Identical mutations within C1 domain (Arg2090, Lys2092 and Phe2093) reduce immunogenicity of FVIII as well as its affinity to LRP. However, as mentioned previously the C1 domain also contributes to the binding of FVIII to phospholipids membranes, a process indispensable for cofactor function of FVIII. Therefore, novel therapeutics designed based on modification of the C1 domain need to be monitored for their effect on cofactor activity. Chromogenic and clotting (data not shown) activity of the C1-domain variant presented and tested in chapter 4 were identical to that of wild-type FVIII; however, others have shown that similar amino acid substitutions in the C1 domain reduced clotting activity. Additional studies are therefore required to verify physiological activity of our C1 domain variant in a clinically-relevant in vivo set-up.

Dendritic cells and modulation of FVIII-specific immune responses
Dendritic cells play a pivotal role in orchestrating the immune system. They are the primary antigen-presenting cells capable of priming naive, antigen-specific T cells, process which leads to formation of subsequent B-cell responses. After encountering proinflammatory stimuli, DCs undergo a maturation process which results in up-regulation of MHC class II and co-stimulatory molecules like CD80, CD83 and CD86 on the plasma membrane. The recognition of peptide-loaded MHC class II by CD4+ T cells on immature APCs, which are lacking co-stimulatory molecules and present low levels of peptide-loaded MHC class II molecules, results in less responsive, anergic or even regulatory T cells which are associated with immune tolerance. This phenomenon has been exploited by several groups to develop protocols for establishing antigen-specific tolerance. Rapamycin is an immunosuppressive drug acting via repression of the mTOR signaling pathway, resulting in deletion of effector T cells. Treatment with rapamycin results in selective expansion of regulatory T cells which proceeds independently of the mTOR pathway. Dendritic cells exposed to rapamycin present a tolerogenic phenotype – they selectively promote the formation of CD4+FoxP3+ regulatory T cells and induce apoptosis of allogenic T cells. Oral administration of rapamycin, combined with intravenous injection of FVIII, effectively prevented inhibitor formation in FVIII−/− mice, and initiated CD4+CD25+Foxp3+ T cell expansion. Co-administration of FVIII and rapamycin resulted in long-lasting tolerance as shown by the lack of antibody formation following post-rapamycin treatment.
with FVIII. Expansion of regulatory T cells by anti-IL-2/IL-2 complexes has also been shown to suppress anti-FVIII responses in a mouse model for hemophilia A. In another protocol, tolerogenic dendritic cells (tDCs) were modified with foamy virus vector expressing FVIII. Infusion of FVIII expressing tDCs in naive or immunized FVIII-deficient mice resulted in diminished T- and B-cell responses to recombinant FVIII; moreover, adoptive transfer of CD4+ T cells isolated from tolerized mice into secondary recipients resulted in antigen-specific immune suppression. Sule and colleagues showed that IL-10 and TGFβ-conditioned DCs are able to inhibit antibody response in naive FVIII-deficient mice. We have used dexamethasone/vitamin D3 conditioned dendritic cells to modulate FVIII-specific immune response in hemophilia mice. After an initial boost with three weekly doses of FVIII, mice received three intravenous injections of FVIII-pulsed mature DCs (mDCs), tolerogenic DCs (tDCs) or a buffer control. Mice injected with FVIII-pulsed mDCs developed high titers of anti-FVIII antibodies, while no or very low titers were observed for control group or animals injected with tDCs (Figure 2A). However, after additional doses of FVIII, mice injected with both types of DCs showed very high antibody titers, while mice boosted with FVIII only had moderate levels of anti-FVIII antibodies (Figure 2B). Although the tDCs had a suppressive phenotype in contrast to mature DC, upon FVIII administration they actually boosted FVIII-specific response rather than inhibited it (as compared to the group that did not receive any specific treatment). These findings suggest

Figure 2. Influence of FVIII-pulsed mature or dexamethasone/vitamin D3-treated dendritic cells on FVIII-specific antibody titers in primed hemophilia A mice. A. After an initial boost with three weekly doses of FVIII (1 µg each), mice received three intravenous injections of 1x10⁶ FVIII-pulsed mature DCs (mDCs), 1x10⁶ tolerogenic DCs (tDCs) or a buffer control. B. After additional 3 doses of FVIII (1 µg each), mice injected with both types of DCs showed very high antibody titers, while mice boosted with FVIII only had moderate levels of anti-FVIII antibodies. Levels of anti-FVIII antibodies in murine plasma were measured by ELISA as described in chapters 3 and 4. 1 AU corresponds to signal obtained with 1 µg mouse monoclonal antibody CLB-CAg9. Data were analyzed using non-parametric Mann-Whitney U-test.
that DC-based tolerance inducing therapies must be carefully controlled as it might potentially result in enhancement of immune responses. Therefore, additional safety measures should be applied before introducing treatment based on tolerogenic dendritic cells in a clinical set-up.

As shown in this thesis, modulation of dendritic cell function is not the only way to suppress FVIII-specific immune responses. We demonstrated that this effect can be also achieved by modifying a site in FVIII that is responsible for its immune recognition by APCs. This approach has number of potential benefits over traditional, general immunosuppressive protocols, among which antigen-specificity is the most important one. Interestingly, the C1 domain variant of FVIII described in chapter 4 displays impaired binding to LRP, indicated as a major receptor involved in FVIII clearance, with unchanged binding affinity to VWF. Therefore, apart from its lower immunogenicity, it can also potentially display prolonged half-life time in the circulation. As such, it represents a novel direction in development of drugs used in hemophilia treatment – generation of a better, long-living FVIII molecule with decreased immunogenicity. In view of the close link between immunogenicity and half-life, it will also be important to assess potential changes in immunogenicity of newly developed products with an increased half-life.

**B cells and anti-FVIII humoral responses in hemophilia**

The majority of studies included in this thesis focus on the role of dendritic cells in initiation and modulation of anti-FVIII immune responses. Other antigen-presenting cells, such as macrophages or B cells are also capable of presenting antigen-derived peptides in a MHC class II dependent manner. The importance of B cells as antigen-presenting cells in initiation of immune response and priming of naive CD4\(^+\) T cells remains unclear. Although they have been shown to contribute to both processes, their importance in priming CD4\(^+\) T cell response appears to be limited. On the other hand, memory B cells play an essential role in maintaining established antibody responses. Upon re-exposure to the same antigen, they are rapidly re-stimulated to proliferate and differentiate into antibody-secreting plasma cells (ASC) that secrete high-affinity antibodies. Such a rapid increase in antibody titers is observed after the onset of ITI, strongly pointing towards re-stimulation of FVIII-specific memory B cells. In hemophilia A patients suffering from the development of anti-FVIII antibodies, FVIII-specific immune complexes (FVIII-ICs) are formed, which can potentially modulate further responses to this therapeutic molecule. We indeed show that formation of FVIII-ICs results in enhanced antigen uptake by dendritic cells and more efficient priming of FVIII-specific T cell responses (Chapter 5). However, we did not investigate the effects of FVIII-ICs on re-stimulation of memory B cells. Fc\(\gamma\)RIIb is the only Fc\(\gamma\)R on B cells and, as an inhibitory receptor, plays important role in suppression of immune responses. It has been shown that in vitro re-stimulation of FVIII-specific memory B cells with low FVIII concentrations leads to their differentiation into antibody-secreting cells (ASCs), while high doses (>1 \(\mu\)g/ml) inhibit this process. Absence of Fc\(\gamma\)RIIb abolishes the differentiation
no matter which concentration of FVIII are used. It would be interesting to see how the presence of FVIII-immune complexes modulates this process, since it is known that FcγRIIb can be co-engaged with BCR with high avidity, which leads to suppression of B cells recognizing cognate antigen.

Final remarks and future directions
Replacement therapy comprising regular injections with either plasma-derived or recombinant FVIII remains the major treatment used for hemophilia patients. Unfortunately, its high cost hampers its availability for many patients. Moreover, it frequently results in development of inhibitory, anti-FVIII antibodies, rendering the therapy ineffective. Therefore, design of long-lived, less immunogenic FVIII remains the most important goal for future studies in the field of hemophilia. In chapter 4 we present novel FVIII molecule characterized by reduced immune recognition, diminished binding to the major clearance receptor – LRP, and unaffected binding to VWF. However, further studies need still to prove its use in clinical set-up. Moreover, the presented FVIII C1 domain variant is still recognized and endocytosed by antigen-presenting cells. In order to further improve it, we still need to increase our knowledge on what drives the endocytosis of FVIII by antigen-presenting cells. Furthermore, since only part of the severe patients develop anti-FVIII antibodies, it is also important to fully understand the mechanism underlying FVIII immunogenicity. Is it only the structure that defines it? Is there an additional trigger involved? Assuming potential mimicry between epitopes exposed by FVIII and pathogens, would pathogen-directed antibodies bind infused, circulating FVIII and could such immune complex formation be partially responsible for induction of immune response against this therapeutic molecule? Those and many other questions still need to be answered in order to further improve current treatment for hemophilia A.
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