Immune recognition and processing of blood coagulation factor VIII by antigen-presenting cells

Simon van Haren

cover: Structural representation of peptide $FVIII_{594-604}$ presented on an MHC class II complex consisting of HLA-DRB1*1101 and HLA-DRA*0101, as described in Chapter 7. This peptide:MHC complex was built using the MHCsim webserver (http://igrid-ext.cryst.bbk.ac.uk/MHCsim/) and depicted using the program pymol.

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Immuun herkenning en verwerking van bloedstollingsfactor VIII door antigeen-presenterende cellen (met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 22 december 2011 des middags te 12.45 uur

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Chapter 1

General introduction

This thesis describes how coagulation factor VIII (FVIII) is recognized by the immune system. The development of FVIII-neutralizing antibodies (inhibitors) is a severe complication occurring after treatment of hemophilia A patients with intravenously administered FVIII. The incidence of inhibitor formation is approximately 25% in patients with the severe form of hemophilia A. Because regular prophylactic treatment with FVIII is no longer effective in the presence of inhibitors, these patients undergo alternative treatment regimens aimed to induce immunological tolerance towards FVIII. Many genetic and environmental risk factors for inhibitor development have been identified but a complete understanding of the immunological basis for inhibitor formation and of the mechanism behind tolerance induction has not yet been established. In this thesis we focus on the recognition of FVIII by antigen-presenting cells (APCs) and the subsequent interaction between APCs and T cells. These are the two processes that mainly shape the immune response against FVIII. Endocytosis of FVIII by APCs and presentation of FVIII peptides to $CD4^+$ T cells provides a crucial step in the initiation of an immune response against FVIII. In this introductory chapter we reflect on the current knowledge available on the formation of antibodies against FVIII and the different genetic and environmental factors involved in this process.

Hemophilia A

Hemophilia A is a hereditary bleeding disorder caused by a deficiency in coagulation factor VIII (FVIII), resulting in excessive and prolonged bleeding.[1] Patients can be classified into three groups based on the severity of the disease. Severe hemophilia A patients have less than 1% residual FVIII in the circulation, moderate hemophilia A patients between 1% and 5% and patients with FVIII levels between 5% and 25% are classified as mild hemophilia A patients. Treatment of patients with hemophilia A with FVIII concentrates results only in a transient rise in FVIII levels in the circulation. Therefore, frequent infusions of FVIII are needed in order to maintain hemostatic FVIII levels required for correction of the bleeding tendency. The first available form of treatment consisted of intravenous administration of FVIII concentrates derived from human donor plasma.[2] Nowadays, the majority of hemophilia A patients are treated with highly purified FVIII derived either from plasma or from culture supernatants of eukaryotic cells expressing recombinant FVIII. A severe complication that results from repeated FVIII infusions is the development of FVIII-neutralizing antibodies (inhibitors). Inhibitor formation occurs in approximately 25% of patients with severe hemophilia A and in 5% of mild or moderate hemophilia A patients.[4] These antibodies inhibit the cofactor function of FVIII, rendering patients unresponsive to FVIII suppletion therapy.[3] Therefore, alternative treatment strategies are necessary for these patients to maintain hemostasis and remove inhibitors. Bleeding episodes in patients with inhibitors are treated with activated prothrombin complex concentrates (APCC)

or recombinant FVIIa; administration of activated FVII and activated clotting factors in APCC restores hemostasis through their ability to directly activate the clotting system downstream of FVIII.[5,6] Other forms of treatment focus on eradication of the inhibitor, which can be achieved by treatment with a B-cell depleting anti-CD20 antibody.[7] Eradication of inhibitors can also be accomplished through immune tolerance induction (ITI).[8] ITI consists of frequent administration of high dosages of FVIII, often in combination with immunosuppressive drugs.[9] This treatment strategy successfully eradicates FVIII inhibitors in the majority of patients with inhibitors. Despite its overall high success-rate tolerance against FVIII cannot be established in 10-20% of cases.[10] Therefore, novel approaches to prevent inhibitor development in hemophilia A are urgently needed. Also novel therapeutic options for treatment of hemophilia A with pre-existing inhibitors would provide a major advancement in hemophilia care. In order to allow for rational design of novel treatment options our current understanding of how inhibitor formation takes place should be further expanded.

Inhibitor formation

Clinical studies have identified several risk factors for the development of inhibitors. As mentioned earlier, the extent of residual activity of endogenous FVIII is inversely correlated with inhibitor formation.[4] Large deletions, nonsense mutations or intron 22 inversions in the gene encoding FVIII, which result predominantly in severe hemophilia A, more often give rise to the development of inhibitors than most missense mutations, which generally result in mild or moderate hemophilia A.[11] Some missense mutations, at position 593, 2150 and 2229, which cause mild hemophilia A, result in a higher incidence in inhibitor formation than most other missense mutations.[12-14] Additional genetic variations outside the gene encoding for FVIII have been identified as risk factors for inhibitor formation as well, such as polymorphisms in the promoter regions of the genes encoding for the cytokines IL-10 and TNF-α.[15,16] A polymorphism in the promoter region of CTLA-4, a receptor involved in the inhibition of T-cell activation, was found to be inversely correlated with inhibitor formation.[17] In a study including a larger group of patients, the correlation of these polymorphisms with inhibitor formation was confirmed.[18] Additionally, this study also showed a correlation between inhibitor formation and HLA alleles DRB1*15 and DQB1*0602. Previous studies have suggested weak associations between HLA haplotypes and inhibitor formation.[12,19,20] The failure of HLA haplotypes to reach a statistically significant correlation with inhibitor formation is most likely due to the large diversity in HLA alleles compared to the relatively small amount of inhibitor patients included in the studies and due to the promiscuity of FVIII peptides in terms of binding to different HLA alleles.[21,22]
Non-genetic risk factors for inhibitor formation, such as treatment intensity, have also been-described. Both an increased amount of exposure to prophylactic FVIII and a decrease in

interval length between exposure days are correlated with an increased risk of inhibitor formation.[23,24] The source of infused FVIII might be associated with inhibitor formation, however, there are conflicting findings about the immunogenicity of the plasma derived or recombinant FVIII.[4,25,26] These clinical observations do not only provide important predictive information but also give useful hints towards how exogenously administered FVIII is recognized by the immune system of hemophilia A patients. IL-10, TNF-α and CTLA-4 are all molecules involved in cross-talk between antigen-presenting cells (APCs) and T cells during a humoral immune response. The first evidence that inhibitor formation is a $CD4^+$ T-cell dependent process came from the observation that in hemophilia A patients infected with HIV the inhibitors disappeared in association with a reduction in $CD4^+$ T cells.[27] The importance of APC-T cell interactions was confirmed in studies using hemophilic mice. Blockade of interactions between CD40 on APCs and CD154 on T cells diminishes inhibitor formation in hemophilic mice, as does blockade of the interaction between CD28 on T cells and CD80 or CD86 on APCs.[28,29]

Initiation of anti-FVIII antibodies

The first step in the initiation of an immune response against FVIII is recognition of FVIII by APCs. Intravenously administered FVIII is internalized by APCs residing in the spleen.[30] *In vitro* analysis using human dendritic cells as model APCs show that the endocytosis of FVIII is receptor-mediated. The endocytosis was hypothesized to be mediated through the interaction of the Macrophage Mannose Receptor (MR) with sugar moieties on the FVIII molecule.[31] Following endocytosis, FVIII is processed into small peptides which are subsequently loaded on MHC class II molecules for presentation to FVIII-specific $CD4^+$ T cells. Different aspects contributing to the endocytosis, processing and subsequent presentation of FVIII are reviewed more elaborately in Chapter 2. A simplified representation of these processes is depicted in Figure 1. Analysis of $CD4^+$ T cells derived from hemophilia A patients has revealed that T-cell responses against FVIII are often of polyclonal nature and directed against multiple domains.[31,32] The activation of these T cells requires additional signals provided by the APC. These signals are membrane-associated interactions between molecules such as CD40, CTLA-4, CD80, CD83 and CD86 on the plasma membrane of the APC and CD28 and CD154 on the T cell. In combination with these interactions, the APC signals to T cells via cytokines such as IL-12 or IL-10. The combination of signals determines the direction into which the activated T cell differentiates. T helper 1 (Th1) cells generally induce a cellular immune response, Th2 a humoral response and regulatory T cells are able to induce tolerance by suppressing B- and T-cell responses. Ultimately, these FVIII-specific T cells are able to activate FVIII-specific B cells and induce affinity maturation and class-switching of immunoglobulin genes in B cells. As a result, anti-FVIII antibody-secreting plasma cells and circulating FVIII-specific memory B cells aregenerated, which are able to produce antibodies upon re-exposure to FVIII.[33,34] Similar to anti-FVIII T-cell responses, FVIII-specific B-cell responses are of polyclonal origin,

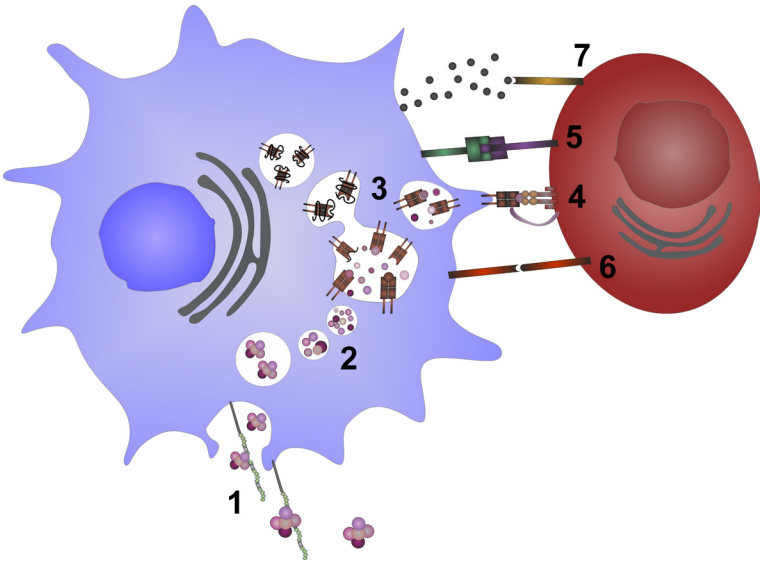

Figure 1. Interactions between APCs and T cells upon recognition of FVIII.
Intravenous administration of FVIII to hemophilia A patients leads to recognition of FVIII by APCs, which is followed by the presentation of FVIII peptides to FVIII-specific T cells. **1.** Endocytosis of FVIII. **2.** Degradation FVIII into small peptides. **3.** Loading of MHC class II with FVIII peptides. **4.** peptide-loaded MHC complexes are presented on the plasma membrane to $CD4^+$ T cells. **5.** Activation of $CD4^+$ T cells via the interaction of CD40 on APCs and CD154 on T cells. **6.** Co-stimulation of $CD4^+$ T cells via the interaction of CD80, CD83 or CD86 on APCs with CD28 on T cells. **7.** Additional signaling of the APC to the $CD4^+$ T cell via cytokines such as IL12 and IL10.

directed against multiple epitopes.[3] Exceptions to this are B-cell responses in mild/moderate hemophilia A patients. These patients have residual levels of endogenous FVIII, containing a mutation, to which they are tolerant. Treatment of these patients with FVIII therefore does not introduce a foreign molecule to the immune system, but rather an alternative molecule which is only one amino acid different from endogenous FVIII. Anti-FVIII antibodies in these patients have been shown to be directed against these newly introduced amino acids.[12,35] In general, FVIII-neutralizing antibodies bind to antigenic sites present on the FVIII molecule, rendering it less active in procoagulant activity. Antibodies directed against the A2 and A3 domain can disrupt the interaction of FVIII with FIXa.[36] Antibodies directed against the C1 or C2 domain are able to interfere with the binding of FVIII to negatively charged phospholipids[36,37], but also with the interaction with von Willebrand Factor.[12] Besides directly interfering with the interaction between FVIII and other components of the coagulation cascade, anti-FVIII antibodies can also indirectly interfere with the coagulation cascade by increasing the clearance rate of FVIII from the circulation.[38] This clearance is mediated by Fc-receptors and therefore dependent on

the antibody subclass. FVIII inhibitors are of the IgG subclass. There is a strong preference for IgG1 and IgG4 isotypes, although IgG2 and IgG3 isotypes can also be present, but usually less abundantly.[39,40] The distribution of inhibitor isotypes can alter during the course of treatment. Patients successfully undergoing ITI are generally characterized by an increased ratio of IgG1 molecules over IgG4 molecules, whereas this ratio often changes to a higher ratio of IgG4 molecules in patients that respond poorly to ITI.[41]

Scope of this thesis

Our current knowledge about how and when the formation of inhibitors takes place and what the effect of ITI is on the immune system of hemophilia A patient is very descriptive. Several risk factors have been identified and antibodies have been characterized in terms of antibody subclass and epitope recognition. An advantage is that some of this information can be directly translated to clinical practice, but they provide minimal information about the basic features of the immune system when dealing with FVIII. Studies using hemophilic mice have provided much information about how the immune system responds to FVIII, regarding the APC cell types involved[29], the development of T cells[42] and requirements for the induction of tolerance.[43] The aim of this thesis is to provide more insight into the mechanism of recognition of FVIII by the human immune system. In Chapter 3 we have re-examined the role of several different endocytic receptors on antigen-presenting cells in the endocytosis of FVIII and we report that the endocytosis of FVIII can be diminished by shielding of a part of the C1 domain with antibody KM33. This effect was confirmed *in vivo*. A single administration of KM33 into hemophilic mice was able to protect these mice from inhibitor formation after 3 subsequent administrations of FVIII. In Chapter 4 we follow up on these findings, describing the construction of a FVIII variant with C1 domain mutations corresponding to the binding site of KM33. We show that this variant has a significantly reduced immunogenicity, while retaining procoagulant activity. In Chapter 5 we have developed a method to identify which regions of the FVIII molecule are presented on MHC class II by human dendritic cells. The findings in this chapter indicate that, although the presentation of FVIII peptides is dependent on donor HLA type, several HLA-promiscuous regions are commonly presented. Chapter 6 describes that presentation of FVIII peptides on MHC class II is not only dependent on HLA type, but can be regulated at different levels. Macrophages appear less efficient in the presentation of FVIII peptides when compared to dendritic cells. The presence of anti-FVIII antibodies or the cytokine milieu during endocytosis of FVIII can also have profound effects on the presentation of FVIII peptides. Finally, we used recombinant HLA-DR molecules loaded with FVIII peptides to identify and characterize FVIII-specific CD4+ T cells in two mild hemophilia A patients. We anticipate that the observations made in this thesis will not only contribute to increased understanding of the immunobiology of inhibitor formation, but will also provide useful indications on how to improve treatment of hemophilia A patients by rational design of FVIII variants with a decreased immunogenicity.

References

1. Mannucci PM, Tuddenham EG. The hemophilias--from royal genes to gene therapy. *N Engl J Med*. 2001;344(23):1773-1779.
2. Pool JG, Shannon AE. Production of high-potency concentrates of antihemophilic globulin in a closed-bag system. *NEnglJ Med*. 1965;273(27):1443-1447.
3. Lollar P. Pathogenic antibodies to coagulation factors. Part one: factor VIII and factor IX. *J Thromb Haemost*. 2004;2(7):1082-1095.
4. Scharrer I, Bray GL, Neutzling O. Incidence of inhibitors in haemophilia A patients--a review of recent studies of recombinant and plasma-derived factor VIII concentrates. *Haemophilia*. 1999;5(3):145-154.
5. Hilgartner M, Aledort L, Andes A, Gill J. Efficacy and safety of vapor-heated anti-inhibitor coagulant complex in hemophilia patients. FEIBA Study Group. *Transfusion*. 1990;30(7):626-630.
6. Key NS, Aledort LM, Beardsley D, et al. Home treatment of mild to moderate bleeding episodes using recombinant factor VIIa (Novoseven) in haemophiliacs with inhibitors. *Thromb Haemost*. 1998;80(6):912-918.
7. Moschovi M, Aronis S, Trimis G, Platokouki H, Salavoura K, Tzortzatou-Stathopoulou F. Rituximab in the treatment of high responding inhibitors in severe haemophilia A. *Haemophilia*. 2006;12(1):95-99.
8. Brackmann HH, Gormsen J. Massive factor-VIII infusion in haemophiliac with factor-VIII inhibitor, high responder. *Lancet*. 1977;2(8044):933.
9. Mariani G, Siragusa S, Kroner BL. Immune tolerance induction in hemophilia A: a review. *SeminThrombHemost*. 2003;29(1):69-76.
10. Goodeve AC, Peake IR. The molecular basis of hemophilia A: genotype-phenotype relationships and inhibitor development. *SeminThrombHemost*. 2003;29(1):23-30.
11. Bril WS, MacLean PE, Kaijen PH, et al. HLA class II genotype and factor VIII inhibitors in mild haemophilia A patients with an Arg593 to Cys mutation. *Haemophilia*. 2004;10(5):509-514.
12. Jacquemin M, Benhida A, Peerlinck K, et al. A human antibody directed to the factor VIII C1 domain inhibits factor VIII cofactor activity and binding to von Willebrand factor. *Blood*. 2000;95(1):156-163.
13. Hay CR. Factor VIII inhibitors in mild and moderate-severity haemophilia A. *Haemophilia*. 1998;4(4):558-563.
14. Astermark J, Oldenburg J, Pavlova A, Berntorp E, Lefvert AK. Polymorphisms in the IL10 but not in the IL1beta and IL4 genes are associated with inhibitor development in patients with hemophilia A. *Blood*. 2006;107(8):3167-3172.
15. Astermark J, Oldenburg J, Carlson J, et al. Polymorphisms in the TNFA gene and the risk of inhibitor development in patients with hemophilia A. *Blood*. 2006;108(12):3739-3745.
16. Astermark J, Wang X, Oldenburg J, Berntorp E, Lefvert AK. Polymorphisms in the CTLA-4 gene and inhibitor development in patients with severe hemophilia A. *J Thromb Haemost*. 2007;5(2):263-265.
17. Pavlova A, Delev D, Lacroix-Desmazes S, et al. Impact of polymorphisms of the MHC class II, IL-10, TNF-a and CTLA-4 genes on inhibitor development in severe hemophilia A. *J Thromb Haemost*. 2009;7(12):2006-2015.

18. Oldenburg J, Picard JK, Schwaab R, Brackmann HH, Tuddenham EG, Simpson E. HLA genotype of patients with severe haemophilia A due to intron 22 inversion with and without inhibitors of factor VIII. *ThrombHaemost*. 1997;77(2):238-242.
19. Hay CR, Ollier W, Pepper L, et al. HLA class II profile: a weak determinant of factor VIII inhibitor development in severe haemophilia A. UKHCDO Inhibitor Working Party. *ThrombHaemost*. 1997;77(2):234-237.
20. Jones TD, Phillips WJ, Smith BJ, et al. Identification and removal of a promiscuous CD4+ T cell epitope from the C1 domain of factor VIII. *J Thromb Haemost*. 2005;3(5):991-1000.
21. van Haren SD, Herczenik E, Ten Brinke A, Mertens K, Voorberg J, Meijer AB. HLA-DR-presented peptide-repertoires derived from human monocyte-derived dendritic cells pulsed with blood coagulation factor VIII. *Mol Cell Proteomics*. 2011. ;10(6):M110 002246.
22. Gouw SC, van den Berg HM, le Cessie S, van der Bom JG. Treatment characteristics and the risk of inhibitor development: a multicenter cohort study among previously untreated patients with severe hemophilia A. *J Thromb Haemost*. 2007;5(7):1383-1390.
23. Eckhardt CL, Menke LA, van Ommen CH, et al. Intensive peri-operative use of factor VIII and the Arg593-->Cys mutation are risk factors for inhibitor development in mild/moderate hemophilia A. *J Thromb Haemost*. 2009;7(6):930-937.
24. Peerlinck K, Arnout J, Di Giambattista M, et al. Factor VIII inhibitors in previously treated haemophilia A patients with a double virus-inactivated plasma derived factor VIII concentrate. *Thromb Haemost*. 1997;77(1):80-86.
25. Gouw SC, van der Bom JG, Auerswald G, Ettinghausen CE, Tedgard U, van den Berg HM. Recombinant versus plasma-derived factor VIII products and the development of inhibitors in previously untreated patients with severe hemophilia A: the CANAL cohort study. *Blood*. 2007;109(11):4693-4697.
26. Ragni MV, Bontempo FA, Lewis JH. Disappearance of inhibitor to factor VIII in HIV-infected hemophiliacs with progression to AIDS or severe ARC. *Transfusion*. 1989;29(5):447-449.
27. Qian J, Burkly LC, Smith EP, et al. Role of CD154 in the secondary immune response: the reduction of pre-existing splenic germinal centers and anti-factor VIII inhibitor titer. *EurJImmunol*. 2000;30(9):2548-2554.
28. Qian J, Collins M, Sharpe AH, Hoyer LW. Prevention and treatment of factor VIII inhibitors in murine hemophilia A. *Blood*. 2000;95(4):1324-1329.
29. Navarrete A, Dasgupta S, Delignat S, et al. Splenic marginal zone antigen presenting cells are critical for the primary allo-immune response to therapeutic factor VIII in hemophilia A. *J Thromb Haemost*. 2009.
30. Dasgupta S, Navarrete AM, Bayry J, et al. A role for exposed mannosylations in presentation of human therapeutic self-proteins to CD4+ T lymphocytes. *Proc Natl Acad Sci U S A*. 2007;104(21):8965-8970.
31. Reding MT, Okita DK, Diethelm-Okita BM, Anderson TA, Conti-Fine BM. Epitope repertoire of human CD4(+) T cells on the A3 domain of coagulation factor VIII. *J Thromb Haemost*. 2004;2(8):1385-1394.
32. Reding MT, Okita DK, Diethelm-Okita BM, Anderson TA, Conti-Fine BM. Human CD4+ T-cell epitope repertoire on the C2 domain of coagulation factor VIII. *J Thromb Haemost*. 2003;1(8):1777-1784.
33. van Helden PM, Van Haren SD, Fijnvandraat K, van den Berg HM, Voorberg J. Factor VIII-specific B cell responses in haemophilia A patients with inhibitors. *Haemophilia*. 2010;16(102):35-43.

34. Hausl C, Maier E, Schwarz HP, et al. Long-term persistence of anti-factor VIII antibody-secreting cells in hemophilic mice after treatment with human factor VIII. *Thromb Haemost*. 2002;87(5):840-845.
35. Bril WS, Turenhout EA, Kaijen PH, et al. Analysis of factor VIII inhibitors in a haemophilia A patient with an Arg593-->Cys mutation using phage display. *Br J Haematol*. 2002;119(2):393-396.
36. Ananyeva NM, Lacroix-Desmazes S, Hauser CA, et al. Inhibitors in hemophilia A: mechanisms of inhibition, management and perspectives. *Blood Coagul Fibrinolysis*. 2004;15(2):109-124.
37. Meems H, Meijer AB, Cullinan DB, Mertens K, Gilbert GE. Factor VIII C1 domain residues Lys 2092 and Phe 2093 contribute to membrane binding and cofactor activity. *Blood*. 2009.
38. Ling M, Duncan EM, Rodgers SE, Street AM, Lloyd JV. Low detection rate of antibodies to non-functional epitopes on factor VIII in patients with hemophilia A and negative for inhibitors by Bethesda assay. *JThrombHaemost*. 2003;1(12):2548-2553.
39. Fulcher CA, de Graaf MS, Zimmerman TS. FVIII inhibitor IgG subclass and FVIII polypeptide specificity determined by immunoblotting. *Blood*. 1987;69(5):1475-1480.
40. Reding MT, Lei S, Lei H, Green D, Gill J, Conti-Fine BM. Distribution of Th1- and Th2-induced anti-factor VIII IgG subclasses in congenital and acquired hemophilia patients. *Thromb Haemost*. 2002;88(4):568-575.
41. Van Helden P, Kaijen PH, Mauser-Bunschoten EP, Fischer K, HM VDB, Voorberg J. Domain specificity of factor VIII inhibitors during immune tolerance induction in patients with haemophilia A. *Haemophilia*. 2010.
42. Waters B, Qadura M, Burnett E, et al. Anti-CD3 prevents factor VIII inhibitor development in hemophilia A mice by a regulatory CD4+CD25+-dependent mechanism and by shifting cytokine production to favor a Th1 response. *Blood*. 2009;113(1):193-203.
43. Qadura M, Othman M, Waters B, et al. Reduction of the immune response to factor VIII mediated through tolerogenic factor VIII presentation by immature dendritic cells. *J Thromb Haemost*. 2008;6(12):2095-2104.

Chapter 2

Requirements for immune recognition and processing of factor VIII by antigen-presenting cells

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Blood Reviews. In press

Abstract

Generation of inhibitory antibodies upon repeated FVIII infusion represents a major complication in hemophilia care. Professional antigen-presenting cells (APCs) are crucial for orchestration of humoral immune responses. APCs are capable of internalizing soluble as well as particulate antigens through various mechanisms resulting in loading of antigen-derived peptides on MHC class I or II for presentation to T cells. This review highlights how FVIII is recognized and processed by APCs. The significance and contribution of candidate receptors involved in FVIII uptake by APC is discussed. Recent findings defining the repertoire of FVIII-peptides presented on MHC class II are addressed. Studies in murine models of hemophilia A suggest that modulation of APC function can reduce inhibitor formation. Based on this we anticipate that modulation of FVIII uptake by APCs may yield novel therapeutic approaches for treatment or prevention of inhibitor formation in patients with hemophilia A.

Introduction

Coagulation factor VIII (FVIII) deficiency results in hemophilia A, which is one of the most prevalent bleeding disorders. It is associated with joint and muscle bleedings and excessive hemorrhages after injury or surgery.[1] Conventional treatment consists of frequent intravenous administration of FVIII. However, such treatment can result in the formation of neutralizing antibodies (inhibitors) that inhibit the cofactor function of FVIII.[2] The development of inhibitors only occurs in approximately 5% of mild or moderate hemophilia A patients, and in 25% of severe hemophilia A patients. The extent of residual activity of endogenous FVIII is inversely correlated with the development of inhibitors.[3] Why only a fraction of patients generate antibodies against FVIII is poorly understood. Both treatment-related and genetic risk factors have been shown to contribute to inhibitor development in hemophilia A. Intensive treatment episodes in the context of injury have been shown to present an important risk factor for inhibitor development.[4] Apart from FVIII gene mutations, such as large gene deletions, gene inversions and stop mutations[5,6], polymorphisms within the IL-10 [7] and TNFA gene[8] have been associated with inhibitor development. Interestingly, a C/T polymorphism in the promoter region of the CTLA-4 gene[9] was overrepresented in hemophilia A patients without inhibitors suggesting a protective role of this SNP in inhibitor formation. The source of infused FVIII products has been linked to inhibitor development, however, there are conflicting findings about the immunogenicity of recombinant versus plasma derived FVIII.[3,10,11] Results from a large multi-center cohort study suggest that the risk of inhibitor formation for plasma-derived FVIII is not clearly lower when compared to recombinant products.[3,10,11]

Most inhibitors are high affinity IgG molecules, predominantly IgG1 and IgG4.[12-14] The formation of such inhibitory IgG molecules requires FVIII-specific $CD4^+$ T-cell help.[12,15] Endocytosis of FVIII by antigen-presenting cells (APCs) and subsequent presentation of FVIII peptides on MHC class II molecules on the surface of APCs is required to trigger the activation of FVIII-specific T cells. In the past years, several studies have addressed how FVIII is processed by APCs. Nevertheless, the exact mechanism of FVIII endocytosis and presentation by APCs remains unclear. Understanding the recognition of FVIII by APCs and subsequent activation of FVIII-specific T cells is crucial for development of novel strategies for treatment or prevention of inhibitor formation in hemophilia A. In this review we address key functions of APCs concerning the formation of FVIII inhibitors: endocytosis of FVIII, intracellular processing, antigen-presentation on MHC class II and activation of $CD4^+$ T cells.

Recognition of FVIII by antigen-presenting cells

Professional antigen-presenting cells (APCs) include macrophages, B-lymphocytes and dendritic cells that are specialized to internalize soluble and particulate antigens and present antigen-derived peptides on MHC class II or MHC class I molecules. Uptake of a soluble antigen can occur through a number of mechanisms that includes endocytosis via clathrin coated pits, caveolae or macropinocytosis[16]. Recent findings suggest that receptor-dependent intracellular sorting of antigens by APCs into different subsets of endosomal vesicles determines whether peptides are presented on MHC class II or cross-presented on MHC class I.[17,18]
FVIII is internalized both *in vivo* and *in vitro* by human dendritic cells (DCs) and human and murine macrophages[19-21], which is followed by the activation of FVIII-specific $CD4^+$ T cells. Subsequent to its uptake, FVIII is processed intracellularly by DCs and efficiently presented on MHC class II.[22] *In vitro*, endocytosis of FVIII is mediated by bivalent ion-dependent receptors, since it is inhibited by the addition of EDTA.[23] FVIII contains multiple glycans that might be targets for C-type lectin receptors.[24] Indeed, two mannose-ending glycans, linked to Asn^{239} in the heavy chain and Asn^{2118} in the light chain, have been proposed to interact with the macrophage mannose receptor (MR).[23] Blockage of mannose-mediated interactions with mannan resulted in a partial reduction of FVIII endocytosis by DCs and decreased T-cell activation. In contrast to this finding, siRNA-mediated knockdown of MR on DCs did not affect FVIII endocytosis.[25] This implies that additional receptors can mediate internalization of FVIII. LDL receptor family members and LDL receptor-related protein (LRP) are essential for FVIII clearance.[26,27] Moreover, both LRP and MR have been described to target ligands to the MHC class II processing and presentation pathway.[28,29] However, despite its important role in the clearance of FVIII, LRP does not contribute to the internalization of FVIII by DCs.[25,30] As the affinities of LRP and MR towards FVIII are relatively low, it is possible that prior to its transfer to endocytic receptors and its final internalization, a preconcentration step is needed for FVIII on the cell surface of APCs. Heparan sulfate proteoglycans (HSPGs) have been shown to assist in LRP-mediated FVIII catabolism.[31] This raises the possibility that HSPGs also contribute to the internalization of FVIII by APCs. As yet the mechanism of FVIII endocytosis by APCs remains unidentified. However, recent findings from our group have shown that an anti-C1 domain antibody prevents FVIII endocytosis by APCs *in vitro* and *in vivo*.[25] We anticipate that this observation will help to identify determinants on FVIII involved in its uptake by APCs.

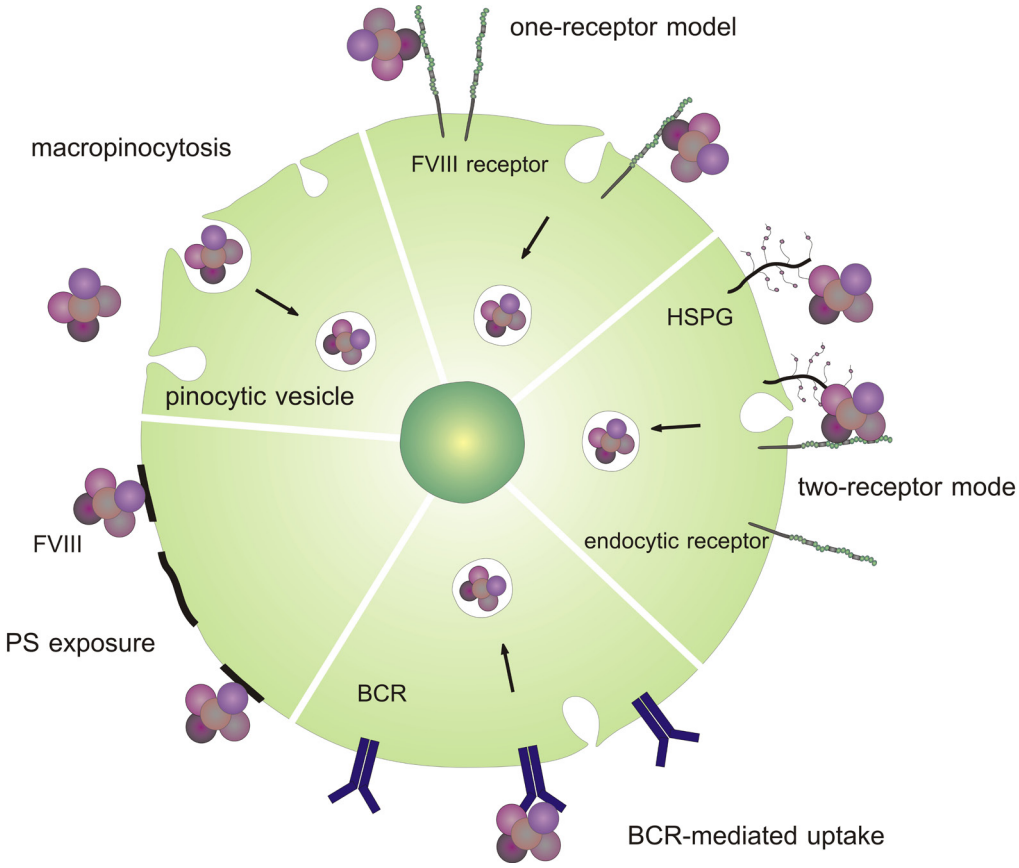

Figure 1. Mechanisms for binding and endocytosis of FVIII by antigen-presenting cells
Fluid phase endocytosis (*macropinocytosis*) is an unsaturable mechanism by which soluble macromolecules such as FVIII can be captured by antigen-presenting cells. The *one-receptor model* represents the machinery driven by a single antigen-specific receptor that is capable of both interacting with FVIII and initiating its endocytosis. The *two-receptor model* on the other hand includes two subsequent mechanisms. The first receptor, like members of heparan sulfate proteoglycans (HSPG), can effectively preconcentrate FVIII on the cell surface. Then FVIII is transferred to a second receptor that endocytoses its ligand. B cells are unique antigen-presenting cells that have ability to internalize highly specific ligands through their cell-surface associated immunoglobulin-like *B-cell receptor (BCR)-mediated uptake.* Both macropinocytosis and receptor-mediated endocytosis are capable of assisting MHC class II-restricted presentation of internalized FVIII. *Phosphatidylserine (PS) exposure* on the outer leaflet of the cell membrane is one of the features of apoptosis. FVIII complex formation with PS does not trigger endocytosis and subsequent presentation of FVIII-derived peptides on MHC class II molecules.

Processing and presentation of FVIII peptides

The fate of an internalized antigen is mainly dependent on its endocytic route, as shown in Figure 1. The proteolysis of soluble antigens such as FVIII starts in early endosomes.[32] Antigen fragments can be either further degraded in lysosomes or loaded on MHC class II complexes, which occurs predominantly in late endosome-derived compartments often referred to as MHC class II-loading compartments.[33] Because lysosomes contain very little MHC class II, they are unlikely to generate peptide-loaded MHC class II for presentation to $CD4^+$ T cells on the plasma membrane.[34] After encountering pro-inflammatory stimuli, dendritic cells (DCs) undergo a maturation process which results in upregulation of MHC class II and co-stimulatory molecules like CD80, CD83 and CD86 on the plasma membrane.[35] Mature DCs are therefore capable of priming naïve $CD4^+$ T cells. Peptide-loaded MHC class II complexes are also present on the plasma membrane of immature APCs, but much less abundantly. Therefore the recognition of peptide-loaded MHC class II by $CD4^+$ T cells on immature APCs, which are also lacking co-stimulatory molecules, results in less responsive, anergic or even regulatory T cells which are associated with immune tolerance.[36,37]

$CD4^+$ T cells recognize antigen derived peptides presented in the context of a particular MHC class II heterodimer of a specific haplotype. Weak associations have been described for human leukocyte antigen (HLA) haplotypes DRB1*1501, DQB1*0602, and DQA1*0102 and formation of FVIII inhibitors.[38,39] Using a mass-spectrometry approach, we have recently shown that numerous FVIII peptides can be presented on MHC class II, some of which were previously shown to be recognized by $CD4^+$ T cells.[22] In addition to FVIII peptides that are donor specific, our study reveals a number of promiscuous FVIII peptides that were presented by multiple MHC II alleles (Figure 2). The fact that a large number of FVIII-derived peptides can be presented on MHC class II is in agreement with the polyclonal nature of FVIII-specific T-cell responses in patients with severe hemophilia A.[40,41] It also provides a rationale for the observation that inhibitor formation is not strongly linked to a particular MHC class II allele. Interestingly, T-cell responses in mild and moderate hemophilia A patients, who have residual endogenous FVIII in the circulation, are often directed against a single epitope around the area of the mutation.[42-44] FVIII-specific $CD4^+$ T cells from a mild hemophilia A patient with an Arg^{2150} to His substitution were directed against a peptide encompassing the area of the mutation.[44] The Arg^{2150}-containing peptide can bind to multiple MHC class II alleles thus providing an explanation for the high prevalence of inhibitor formation in mild hemophilia A patients with this particular gene defect. These findings emphasize the potential importance of predicting affinities of different MHC class II haplotypes towards FVIII peptides in moderate and mild hemophilia A patients. However, in case of severe hemophilia, identification of promiscuous FVIII peptides and introduction of FVIII modifications in these areas in order to reduce their ability to fit in an MHC class II binding groove has been proposed as a means to reduce the intrinsic immunogenicity of FVIII.[45]

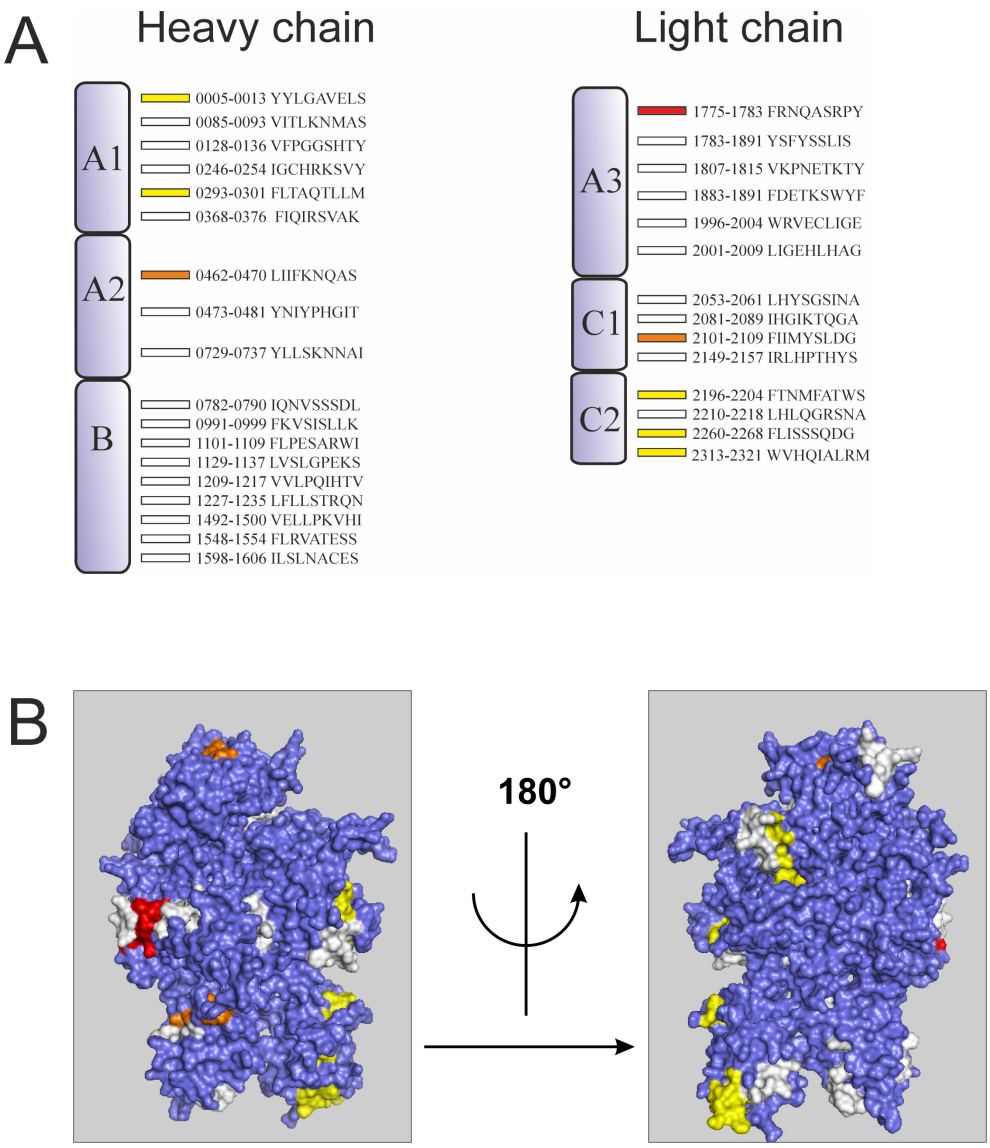

Figure 2: Distribution of FVIII peptides presented on MHC class II.
(A) Different peptides are presented by different donors. Results displayed in this figure were obtained from single experiments from four unrelated donors as described previously.[22] FVIII-derived MHC class II-presented peptides are displayed as rectangles. Peptides presented by only one donor are indicated as white bars. Indicated in yellow are peptides that are common between two donors. The orange color indicates FVIII peptides that were found in three donors and the red sequences were presented in all four donors. **(B)** The different domains and the location of the peptides are depicted in the crystal structure of FVIII.[69] B domain-derived peptides are not depicted, since the B domain is absent in the three-dimensional structure of FVIII.

Co-stimulation/danger signals

APCs increase the expression of co-stimulatory receptors after recognition of pathogen-associated molecular patterns (PAMPs). *In vitro*, FVIII alone or FVIII in complex with VWF does not trigger the upregulation of co-stimulatory receptors by DCs, and therefore is not capable of initiating T-cell activation.[46] *In vivo*, however, adjuvant-free administration of FVIII can lead to the formation of inhibitory antibodies. The biological activity of FVIII appears to be essential for this process, more specifically, its ability to enhance thrombin generation, has been proposed to activate DCs, finally resulting in induction of FVIII-specific T-cell responses (Figure 3).[47] 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*.[48,49] CD40/CD40L ligation provides a key event to induce humoral responses against antigens[50], furthermore, blockage of CD40/CD40L interaction leads to long-lasting tolerance in mice.[51,52] Although 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.[49]

A number of recent studies have described modulatory mechanisms to reduce anti-FVIII responses either through preventing FVIII uptake or by suppressing activation of APCs. VWF has been shown to inhibit activation of FVIII-specific T cells by blocking its uptake by DCs[19], suggesting that VWF prevents the entry of FVIII in APCs by engaging epitope(s) that are key for interactions with cell surface receptors on APCs. Complex formation of FVIII with phosphatidylserine (PS) liposomes resulted in reduced antibody formation against FVIII in hemophilia A mice.[53] PS associated with apoptotic cells is known to induce anti-inflammatory responses in APCs[54]. Moreover, PS liposomes reduced the maturation, pro-inflammatory cytokine production and T-cell priming of APCs.[54] Since complex formation between FVIII and phospholipid molecules, which is essential for FVIII activation[55], is mediated by residues located in the C1 and C2 domains of FVIII[56,57] (Table 1), a possible other explanation of the observed inhibitory effect is that PS occupies residues of FVIII that are crucial for its endocytosis by APCs. Another possibility to manipulate immune responses against FVIII is to suppress APCs by inhibiting their maturation and lowering the expression of co-stimulatory molecules. *In vivo*, dendritic cells subsets that display a tolerogenic phenotype are found in blood or in lymph nodes.[58] Induction of this tolerogenic phenotype by dexamethasone, vitamin D3, rapamycin or aspirin has been successfully used to prevent immune responses.[59] Rapamycin-conditioned DCs are resistant to maturation and poor in stimulation of allogenic $CD4^+$ T cells, however, they enrich for antigen-specific $FoxP3^+$ regulatory T cells.[60-62] Oral administration of rapamycin, combined with intravenous injection of FVIII, effectively prevented inhibitor formation in $FVIII^{-/-}$ mice[63] and initiated $CD4^+CD25^+Foxp3^+$ T cell expansion. The lack of antibody formation was sustained during additional FVIII injections following rapamycin administration. Given the mild and transient immunosuppressive capacity of rapamycin , co-administration of FVIII with rapamycin may be a feasible treatment strategy in hemophilia A patients to induce tolerance to FVIII.

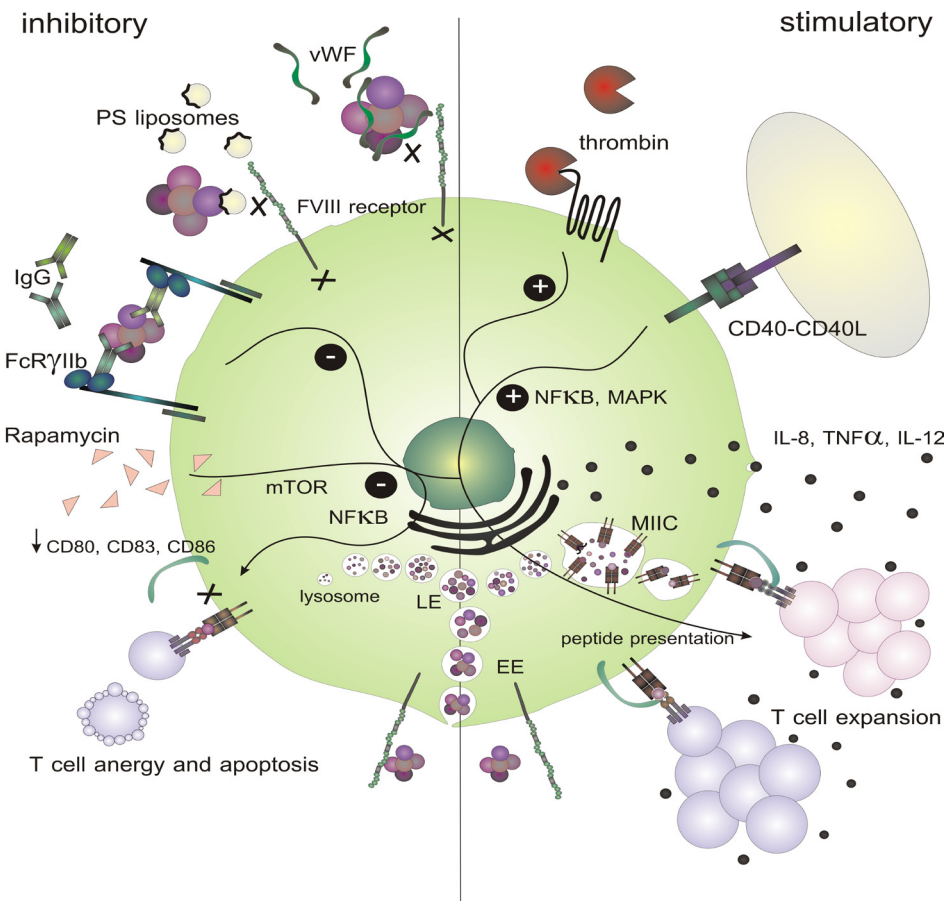

Figure 3. Stimulatory and inhibitory pathways modulate presentation of FVIII on MHC class II
FVIII itself does not trigger the upregulation of co-stimulatory molecules on DCs, therefore it is not capable of initiating T-cell activation and expansion that, besides the interaction of T-cell receptor with peptide-associated MHC class II, also requires a second signal from co-stimulatory molecules like CD80, CD83 or CD86. Thrombin generation has been suggested to provide a "danger signal" that activates antigen-presenting cells. Similarly, association of CD40 with its ligand, CD40L, which is expressed on a wide variety of cells including macrophages, neutrophils, platelets, endothelial cells etc, is another effective mechanism to stimulate the expression of co-stimulatory molecules and production of pro-inflammatory cytokines on APCs through the MAPK pathway and induction of NF-κB translocation to the nucleus. FVIII presentation can be inhibited at many different points. PS liposomes as well as VWF can inhibit FVIII interaction with its receptor on APCs. In hemophilia A patients, FVIII can circulate within immune complexes comprising IgG molecules. Antigen-presenting cells express Fc receptors that are capable of binding to immune complex-associated IgG molecules. Crosslinking of the inhibitory FcγRIIb results in reduced MAPK signaling and prevents excessive activation of the NF-κB pathway. Rapamycin blocks mammalian target of rapamycin (mTOR), which alters the maturation of APCs through downregulation of the expression of co-stimulatory molecules .

Professional APC types and their localization

In vivo, there are three types of professional APCs that may contribute to the induction of an immune response against FVIII, dendritic cells, macrophages and B lymphocytes, which are all able to internalize FVIII and present FVIII peptides on MHC class II. The extent to which each cell type contributes to inhibitor formation is highly dependent on the localization of APCs and their capacity to present FVIII. Given the fact that FVIII is mostly administered intravenously, it is likely that the APCs that are responsible for initiating immune responses against FVIII are either present in the circulation or reside in the spleen. In patients who receive FVIII for the first time splenic DCs or macrophages are probably the most important contributors to FVIII endocytosis and presentation.[21] The importance of APCs residing in the spleen has been confirmed in a study using FVIII$^{-/-}$ mice.[21] The major contributors in terms of FVIII internalization are metallophilic macrophages located in the marginal zone of the spleen.[21] To a lesser extent, MARCO^{+} macrophages and CD11c^{+} DCs also internalized FVIII.[21] However, despite their modest FVIII uptake, DCs might still significantly contribute to inhibitor formation as these cells are more efficient in antigen processing and presentation than macrophages.[64,65] Intravenous infusion delivers FVIII to the spleen, which is the major organ to initiate immune responses.

FVIII-specific memory B cells are present in the circulation of hemophilia A patients with inhibitors.[66] FVIII-specific memory B cells are essential for the rapid production of serum immunoglobulin levels upon re-exposure to infused FVIII. Furthermore, elimination of FVIII-specific memory B cells by high dosage FVIII may contribute to induction of immune tolerance.[67] Tolerance induction through retroviral transduction with retroviral constructs encoding FVIII A2-domain 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.[68] 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.[67,68]

Conclusions and future perspectives

A humoral immune response directed against therapeutic FVIII develops in approximately 25% of severe hemophilia A patients undergoing FVIII replacement therapy. There are multiple factors that can determine the characteristics of the anti-FVIII responses in terms of magnitude, antibody type, duration of the response and tolerance susceptibility. The initial step in the development of FVIII-neutralizing antibodies is recognition of FVIII by APCs. Here, we showed that the course of FVIII recognition and processing by APCs determines the outcome of an anti-FVIII response. Recent years' efforts to identify the endocytic receptor for FVIII on APCs resulted in several candidate receptors. This knowledge could be used to design a FVIII variant which is no longer internalized by APCs but retains its procoagulant activity. Because not all endocytic receptors allow for efficient presentation of their ligand on MHC class II, an interesting alternative would be to engineer FVIII in such a way that its internalization leads to complete degradation rather than presentation. Identification of FVIII-peptide binding properties of individual MHC class II haplotypes provides a useful tool to identify and subsequently modify promiscuous T-cell epitopes thereby reducing the intrinsic immunogenicity of FVIII. Overall, avoiding or altering FVIII recognition by APCs, altering the phenotypic properties of APCs with compounds such as rapamycin or reducing presentation of FVIII peptides on MHC class II have recently emerged as promising, novel approaches for prevention or treatment of inhibitor development in hemophilia A.

	FVIII Domains	residues	Interaction partner	reference
Light Chain	A1 (1-336)	239	Mannose Receptor	Medzihradszky, Anal Chem 1997, 69: 3986–3994 Dasgupta, PNAS, 2007;104(21):8965-8970
	A2 (373-740)	484-509	LRP	Saenko, 1999 JBC, 274, 37685-37692 Ananyeva, BCF, 2008, 19(6):543-55.
		558-565	HSPG	Sarafanov, JBC, 2001, 11970–11979
Heavy Chain	Acidic A3 (a3)	1649-1689	vWF	Saenko, JBC, 1997, 18007–18014
	A3 (1690-2019)	1811-1818	LRP	Bovenschen, JBC, 2003, 9370-9377
	C1 (2020-2172)	2118	Mannose Receptor	Medzihradszky, Anal Chem 1997, 69: 3986–3994 Dasgupta. PNAS. 2007:104(21):8965-8970
		2092-2093	LRP	Meems, Int J Biochem Cell Biol, 2011
	C2 (2173-2332)	C-terminus	LRP	Lenting, 1999 JBC, 274, 23734-23739
		2248-2312	vWF	Shima M, Thr Haem, 1993 69(3):240-6 Saenko, JBC, 1994, 269:11601-11605
		2170-2312	Phosphatidylserine	Shima M, Thr Haem, 1993 69(3):240-6 Foster, Blood, 1990, 75:1999-2004

Table 1. Domain and residue specificity of interactive partners of FVIII

References

1. Mannucci PM, Tuddenham EG. The hemophilias--from royal genes to gene therapy. *N Engl J Med*. 2001;344(23):1773-1779.
2. Lollar P. Pathogenic antibodies to coagulation factors. Part one: factor VIII and factor IX. *J Thromb Haemost*. 2004;2(7):1082-1095.
3. Scharrer I, Bray GL, Neutzling O. Incidence of inhibitors in haemophilia A patients--a review of recent studies of recombinant and plasma-derived factor VIII concentrates. *Haemophilia*. 1999;5(3):145-154.
4. Gouw SC, van den Berg HM, le Cessie S, van der Bom JG. Treatment characteristics and the risk of inhibitor development: a multicenter cohort study among previously untreated patients with severe hemophilia A. *J Thromb Haemost*. 2007;5(7):1383-1390.
5. Schwaab R, Brackmann HH, Meyer C, et al. Haemophilia A: mutation type determines risk of inhibitor formation. *Thromb Haemost*. 1995;74(6):1402-1406.
6. Tuddenham EG, McVey JH. The genetic basis of inhibitor development in haemophilia A. *Haemophilia*. 1998;4(4):543-545.
7. Astermark J, Oldenburg J, Pavlova A, Berntorp E, Lefvert AK. Polymorphisms in the IL10 but not in the IL1beta and IL4 genes are associated with inhibitor development in patients with hemophilia A. *Blood*. 2006;107(8):3167-3172.
8. Astermark J, Oldenburg J, Carlson J, et al. Polymorphisms in the TNFA gene and the risk of inhibitor development in patients with hemophilia A. *Blood*. 2006;108(12):3739-3745.
9. Astermark J, Wang X, Oldenburg J, Berntorp E, Lefvert AK. Polymorphisms in the CTLA-4 gene and inhibitor development in patients with severe hemophilia A. *J Thromb Haemost*. 2007;5(2):263-265.
10. Peerlinck K, Arnout J, Di Giambattista M, et al. Factor VIII inhibitors in previously treated haemophilia A patients with a double virus-inactivated plasma derived factor VIII concentrate. *Thromb Haemost*. 1997;77(1):80-86.
11. Gouw SC, van der Bom JG, Auerswald G, Ettinghausen CE, Tedgard U, van den Berg HM. Recombinant versus plasma-derived factor VIII products and the development of inhibitors in previously untreated patients with severe hemophilia A: the CANAL cohort study. *Blood*. 2007;109(11):4693-4697.
12. Reding MT, Lei S, Lei H, Green D, Gill J, Conti-Fine BM. Distribution of Th1- and Th2-induced anti-factor VIII IgG subclasses in congenital and acquired hemophilia patients. *Thromb Haemost*. 2002;88(4):568-575.
13. van Helden PM, van den Berg HM, Gouw SC, et al. IgG subclasses of anti-FVIII antibodies during immune tolerance induction in patients with hemophilia A. *Br J Haematol*. 2008;142(4):644-652.
14. Fulcher CA, de Graaf MS, Zimmerman TS. FVIII inhibitor IgG subclass and FVIII polypeptide specificity determined by immunoblotting. *Blood*. 1987;69(5):1475-1480.
15. Ragni MV, Bontempo FA, Lewis JH. Disappearance of inhibitor to factor VIII in HIV-infected hemophiliacs with progression to AIDS or severe ARC. *Transfusion*. 1989;29(5):447-449.
16. Trombetta ES, Mellman I. Cell biology of antigen processing in vitro and in vivo. *Annu Rev Immunol*. 2005;23:975-1028.
17. Burgdorf S, Kurts C. Endocytosis mechanisms and the cell biology of antigen presentation. *Curr Opin Immunol*. 2008;20(1):89-95.

18. Lakadamyali M, Rust MJ, Zhuang X. Ligands for clathrin-mediated endocytosis are differentially sorted into distinct populations of early endosomes. *Cell*. 2006;124(5):997-1009.
19. Dasgupta S, Repesse Y, Bayry J, et al. VWF protects FVIII from endocytosis by dendritic cells and subsequent presentation to immune effectors. *Blood*. 2007;109(2):610-612
20. van Schooten CJ, Shahbazi S, Groot E, et al. Macrophages contribute to the cellular uptake of von Willebrand factor and factor VIII in vivo. *Blood*. 2008;112(5):1704-1712.
21. Navarrete A, Dasgupta S, Delignat S, et al. Splenic marginal zone antigen presenting cells are critical for the primary allo-immune response to therapeutic factor VIII in hemophilia A. *J Thromb Haemost*. 2009.
22. van Haren SD, Herczenik E, Ten Brinke A, Mertens K, Voorberg J, Meijer AB. HLA-DR-presented peptide-repertoires derived from human monocyte-derived dendritic cells pulsed with blood coagulation factor VIII. *Mol Cell Proteomics*. 2011 ;10(6):M110 002246.
23. Dasgupta S, Navarrete AM, Bayry J, et al. A role for exposed mannosylations in presentation of human therapeutic self-proteins to CD4+ T lymphocytes. *Proc Natl Acad Sci U S A*. 2007;104(21):8965-8970.
24. Lenting PJ, Christophe OD, Gueguen P. The disappearing act of factor VIII. *Haemophilia*. 2008.
25. Herczenik E, van Haren SD, Wroblewska A, et al. Uptake of blood coagulation factor VIII by dendritic cells is mediated via its C1 domain. *J Allergy Clin Immunol*. 2011. In Press.
26. Schwarz HP, Lenting PJ, Binder B, et al. Involvement of low-density lipoprotein receptor-related protein (LRP) in the clearance of factor VIII in von Willebrand factor-deficient mice. *Blood*. 2000;95(5):1703-1708.
27. Cunningham N, Laffan MA, Manning RA, O'Donnell JS. Low-density lipoprotein receptor-related protein polymorphisms in patients with elevated factor VIII coagulant activity and venous thrombosis. *Blood Coagul Fibrinolysis*. 2005;16(7):465-468.
28. Fischer N, Haug M, Kwok WW, et al. Involvement of CD91 and scavenger receptors in Hsp70-facilitated activation of human antigen-specific CD4(+) memory T cells. *Eur J Immunol*. 2010.
29. Singh SK, Stephani J, Schaefer M, et al. Targeting glycan modified OVA to murine DC-SIGN transgenic dendritic cells enhances MHC class I and II presentation. *Mol Immunol*. 2009.
30. Dasgupta S, Navarrete AM, Andre S, et al. Factor VIII bypasses CD91/LRP for endocytosis by dendritic cells leading to T-cell activation. *Haematologica*. 2008;93(1):83-89.
31. Sarafanov AG, Ananyeva NM, Shima M, Saenko EL. Cell surface heparan sulfate proteoglycans participate in factor VIII catabolism mediated by low density lipoprotein receptor-related protein. *J Biol Chem*. 2001;276(15):11970-11979.
32. Villadangos JA, Ploegh HL. Proteolysis in MHC class II antigen presentation: who's in charge? *Immunity*. 2000;12(3):233-239.
33. Griffin JP, Chu R, Harding CV. Early endosomes and a late endocytic compartment generate different peptide-class II MHC complexes via distinct processing mechanisms. *J Immunol*. 1997;158(4):1523-1532.
34. Harding CV, Geuze HJ. Immunogenic peptides bind to class II MHC molecules in an early lysosomal compartment. *J Immunol*. 1993;151(8):3988-3998.
35. Ten Brinke A, Karsten ML, Dieker MC, Zwaginga JJ, van Ham SM. The clinical grade maturation cocktail monophosphoryl lipid A plus IFNgamma generates monocyte-derived dendritic cells with the capacity to migrate and induce Th1 polarization. *Vaccine*. 2007;25(41):7145-7152.
36. Lutz MB, Kukutsch NA, Menges M, Rossner S, Schuler G. Culture of bone marrow cells in GM-CSF plus high doses of lipopolysaccharide generates exclusively immature dendritic cells which induce alloantigen-specific CD4 T cell anergy in vitro. *Eur J Immunol*. 2000;30(4):1048-1052.

37. Qadura M, Othman M, Waters B, et al. Reduction of the immune response to factor VIII mediated through tolerogenic factor VIII presentation by immature dendritic cells. *J Thromb Haemost*. 2008;6(12):2095-2104.
38. Hay CR, Ollier W, Pepper L, et al. HLA class II profile: a weak determinant of factor VIII inhibitor development in severe haemophilia A. UKHCDO Inhibitor Working Party. *ThrombHaemost*. 1997;77(2):234-237.
39. Oldenburg J, Picard JK, Schwaab R, Brackmann HH, Tuddenham EG, Simpson E. HLA genotype of patients with severe haemophilia A due to intron 22 inversion with and without inhibitors of factor VIII. *ThrombHaemost*. 1997;77(2):238-242.
40. Reding MT, Okita DK, Diethelm-Okita BM, Anderson TA, Conti-Fine BM. Epitope repertoire of human CD4(+) T cells on the A3 domain of coagulation factor VIII. *J Thromb Haemost*. 2004;2(8):1385-1394.
41. Reding MT, Okita DK, Diethelm-Okita BM, Anderson TA, Conti-Fine BM. Human CD4+ T-cell epitope repertoire on the C2 domain of coagulation factor VIII. *J Thromb Haemost*. 2003;1(8):1777-1784.
42. James EA, van Haren SD, Ettinger RA, et al. T-cell responses in two unrelated hemophilia A inhibitor subjects include an epitope at the factor VIII R593C missense site. *J Thromb Haemost*. 2011.
43. James EA, Kwok WW, Ettinger RA, Thompson AR, Pratt KP. T-cell responses over time in a mild hemophilia A inhibitor subject: epitope identification and transient immunogenicity of the corresponding self-peptide. *J Thromb Haemost*. 2007;5(12):2399-2407.
44. Jacquemin M, Vantomme V, Buhot C, et al. CD4+ T-cell clones specific for wild-type factor VIII: a molecular mechanism responsible for a higher incidence of inhibitor formation in mild/moderate hemophilia A. *Blood*. 2003;101(4):1351-1358.
45. Jones TD, Phillips WJ, Smith BJ, et al. Identification and removal of a promiscuous CD4+ T cell epitope from the C1 domain of factor VIII. *J Thromb Haemost*. 2005;3(5):991-1000.
46. Pfistershammer K, Stockl J, Siekmann J, Turecek PL, Schwarz HP, Reipert BM. Recombinant factor VIII and factor VIII-von Willebrand factor complex do not present danger signals for human dendritic cells. *Thromb Haemost*. 2006;96(3):309-316.
47. Skupsky J, Zhang AH, Su Y, Scott DW. A role for thrombin in the initiation of the immune response to therapeutic Factor VIII. *Blood*. 2009;114(21):4741-4748.
48. Qian J, Burkly LC, Smith EP, et al. Role of CD154 in the secondary immune response: the reduction of pre-existing splenic germinal centers and anti-factor VIII inhibitor titer. *EurJImmunol*. 2000;30(9):2548-2554.
49. Reipert BM, Sasgary M, Ahmad RU, Auer W, Turecek PL, Schwarz HP. Blockade of CD40/CD40 ligand interactions prevents induction of factor VIII inhibitors in hemophilic mice but does not induce lasting immune tolerance. *Thromb Haemost*. 2001;86(6):1345-1352.
50. Foy TM, Aruffo A, Bajorath J, Buhlmann JE, Noelle RJ. Immune regulation by CD40 and its ligand GP39. *Annu Rev Immunol*. 1996;14:591-617.
51. Honey K, Cobbold SP, Waldmann H. CD40 ligand blockade induces CD4+ T cell tolerance and linked suppression. *J Immunol*. 1999;163(9):4805-4810.
52. Rossi G, Sarkar J, Scandella D. Long-term induction of immune tolerance after blockade of CD40-CD40L interaction in a mouse model of hemophilia A. *Blood*. 2001;97(9):2750-2757.
53. Purohit VS, Ramani K, Sarkar R, Kazazian HH, Jr., Balasubramanian SV. Lower inhibitor development in hemophilia A mice following administration of recombinant factor VIII-O-phospho-L-serine complex. *J Biol Chem*. 2005;280(18):17593-17600.

54. Hoffmann PR, Kench JA, Vondracek A, et al. Interaction between phosphatidylserine and the phosphatidylserine receptor inhibits immune responses in vivo. *J Immunol*. 2005;174(3):1393-1404.
55. Duffy EJ, Parker ET, Mutucumarana VP, Johnson AE, Lollar P. Binding of factor VIIIa and factor VIII to factor IXa on phospholipid vesicles. *J Biol Chem*. 1992;267(24):17006-17011.
56. Meems H, Meijer AB, Cullinan DB, Mertens K, Gilbert GE. Factor VIII C1 domain residues Lys 2092 and Phe 2093 contribute to membrane binding and cofactor activity. *Blood*. 2009.
57. Novakovic VA, Cullinan DC, Wakabayashi H, Fay P, Baleja JD, Gilbert GE. Membrane binding properties of the factor VIII C2 domain. *Biochem J*.2011;435(1):187-196.
58. Gregori S. Dendritic cells in networks of immunological tolerance. *Tissue Antigens*. 2011;77(2):89-99.
59. Morelli AE, Thomson AW. Tolerogenic dendritic cells and the quest for transplant tolerance. *Nat Rev Immunol*. 2007;7(8):610-621.
60. Hackstein H, Taner T, Zahorchak AF, et al. Rapamycin inhibits IL-4--induced dendritic cell maturation in vitro and dendritic cell mobilization and function in vivo. *Blood*. 2003;101(11):4457-4463.
61. Taner T, Hackstein H, Wang Z, Morelli AE, Thomson AW. Rapamycin-treated, alloantigen-pulsed host dendritic cells induce ag-specific T cell regulation and prolong graft survival. *Am J Transplant*. 2005;5(2):228-236.
62. Turnquist HR, Raimondi G, Zahorchak AF, Fischer RT, Wang Z, Thomson AW. Rapamycin-conditioned dendritic cells are poor stimulators of allogeneic CD4+ T cells, but enrich for antigen-specific Foxp3+ T regulatory cells and promote organ transplant tolerance. *J Immunol*. 2007;178(11):7018-7031.
63. Moghimi B, Sack BK, Nayak S, Markusic DM, Mah CS, Herzog RW. Tolerance Induction to Factor VIII by Transient Co-administration with Rapamycin. *J Thromb Haemost*. 2011.
64. Rodriguez A, Regnault A, Kleijmeer M, Ricciardi-Castagnoli P, Amigorena S. Selective transport of internalized antigens to the cytosol for MHC class I presentation in dendritic cells. *Nat Cell Biol*. 1999;1(6):362-368.
65. Mellman I, Steinman RM. Dendritic cells: specialized and regulated antigen processing machines. *Cell*. 2001;106(3):255-258.
66. van Helden PM, Kaijen PH, Fijnvandraat K, van den Berg HM, Voorberg J. Factor VIII-specific memory B cells in patients with hemophilia A. *J Thromb Haemost*. 2007;5(11):2306-2308.
67. Skupsky J, Zhang AH, Su Y, Scott DW. B-Cell-Delivered Gene Therapy Induces Functional T Regulatory Cells and Leads to a Loss of Antigen-Specific Effector Cells. *Mol Ther*. 2010.
68. Lei TC, Scott DW. Induction of tolerance to factor VIII inhibitors by gene therapy with immunodominant A2 and C2 domains presented by B cells as Ig fusion proteins. *Blood*. 2005;105(12):4865-4870.
69. Ngo JC, Huang M, Roth DA, Furie BC, Furie B. Crystal structure of human factor VIII: implications for the formation of the factor IXa-factor VIIIa complex. *Structure*. 2008;16(4):597-606.

Chapter 3

Uptake of blood coagulation factor VIII by dendritic cells is mediated via its C1 domain

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Abstract

Background: Uptake and processing of FVIII by antigen-presenting cells and subsequent presentation of FVIII-derived peptides to CD4+ T cells directs the immune response to FVIII in patients with hemophilia A. Multiple receptors including mannose receptor (MR) and LDL receptor-related protein-1 (LRP) have been implicated in FVIII uptake.
Objective: This work studies the involvement of receptor candidates in FVIII uptake by dendritic cells. Furthermore, we explore FVIII residues that mediate endocytosis.
Methods: FVIII uptake was performed with human monocyte derived and murine bone marrow-derived dendritic cells. To investigate FVIII endocytosis, competition assays with soluble receptor ligands, binding studies with recombinant receptor fragments, and siRNA-induced gene silencing were performed. Additionally, FVIII targeting monoclonal antibodies KM33 and VK34 were used. To confirm *in vitro* results, hemophilic E17 KO mice were pre-treated with antibodies prior to FVIII injections and anti-FVIII titers were determined.
Results: Upon treatment of DCs with mannan or LRP ligand α2-macroglobulin, we only observed a minor decrease in FVIII internalization. Additionally, siRNA mediated knockdown of LRP, MR or DC-SIGN expression in MDDCs did not prevent FVIII uptake. Binding studies using Fc-chimeras revealed that LRP, DC-SIGN and MR can bind to FVIII; however we did not observe critical role for these receptors in FVIII uptake. Previous studies have shown that human antibodies targeting C1 (KM33) and A2 (VK34) domains of FVIII interfere with binding to endocytic receptors. Preincubation of FVIII with VK34 did not influence FVIII uptake; however, KM33 completely inhibited FVIII endocytosis by both MDDC and BMDC. Accordingly, anti-FVIII antibody titers were greatly reduced following pre-administration of KM33 *in vivo*.
Conclusion: Together, our observations emphasize the physiological significance of KM33-targeted residues within the C1 domain in the uptake of FVIII by DCs *in vitro* and *in vivo*.

Introduction

Immunogenicity of protein therapeutics following repeated exposure remains a major challenge in clinical care. Formation of anti-drug antibodies can severely interfere or neutralize the effect of treatment. Replacement therapy with intravenously administered plasma derived or recombinant coagulation Factor VIII concentrates corrects bleeding tendency and represents the main cure for hemophilia A.[1] Development of neutralizing antibodies (inhibitors) against FVIII occurs in approximately 20-40% of severe hemophilia A patients after replacement therapy, providing a major complication of hemophilia care.[2] The antibody response is heterogeneous with antibodies binding to multiple domains of FVIII.[3,4] To eliminate neutralizing antibodies, immune tolerance induction (ITI) therapy comprising frequent administration of high doses of FVIII is commonly initiated in hemophilia A patients with inhibitors.[5] Subclass analysis revealed that anti-FVIII antibodies are composed of subclass IgG1 and IgG4.[6,7] Clonal analysis revealed that the variable domains of anti-FVIII antibodies are extensively modified by somatic hypermutation.[8,9] Both somatic hypermutation and isotype switching require the presence of antigen specific CD4+ T cells[10], which are enumerated following processing and presentation of FVIII derived peptides on MHC class II molecules on antigen-presenting cells (APCs).[11] Dendritic cells (DCs) are professional APCs that mediate uptake, intracellular processing and presentation of antigen to T cells.[12] Processing of soluble antigens occurs by macrophages and resident DCs present in the spleen.[13] Studies with human DCs have indicated that *in vitro* administration of FVIII does not result in activation and maturation of DCs.[14] A slight increase in CD40 expression was noted upon administration of canine FVIII to bone marrow-derived murine DCs.[15] Human DCs have been reported to internalize FVIII in a macrophage mannose receptor (MR, CD206) dependent manner.[16] Mannose-ending glycans are present in the C1 (Asn^{2118}) and A1 (Asn^{239}) domains of FVIII[17] and provide potential targets for carbohydrate recognition domains of C-type lectin receptors such as MR, DC-SIGN and dectin-2.[18] Although a recent report suggests that LDL-receptor related protein (LRP) is not involved in the uptake of FVIII by dendritic cells[19], LRP is known as one of the main receptors engaged in clearance of FVIII.[19-21] We previously described a panel of antibodies that were obtained from hemophilia patients.[9,22] Amongst these antibodies VK34 and KM33 interfered with FVIII binding to LRP/LDL receptor family members by occupying residues within the A2 and C1 domain, respectively.[23,24] In this study, we show that patient-derived monoclonal antibody KM33 interferes with uptake of FVIII by DCs. Using small interfering RNA we show that FVIII uptake is independent of the presence of LRP, MR and DC-SIGN. Additionally, we show that pre-injection of KM33 prior to FVIII administration prevents antibody formation against FVIII in hemophilic E17 KO mice. Our results suggest that uptake of FVIII by APCs is mediated by an interactive surface in the C1 domain, which does not overlap with a previously binding site of MR.

Materials and Methods

FVIII uptake by DCs

Blood was drawn after written consent from healthy volunteers in accordance with Dutch regulations and following approval from Sanquin Ethical Advisory Board in accordance with the Declaration of Helsinki. Endocytosis of FVIII by dendritic cells was measured as reported previously[25], for details, see Online Repository Materials available at http://www.jacionline.org/.

Binding of FVIII to recombinant human LRP1 Cluster II-Fc, MR CTLD4-7-Fc and DC-SIGN-Fc chimeras

To capture FVIII, C2 domain targeting recombinant human IgG4 antibody EL14[26] (5 µg/ml) was first immobilized on 96 well microtiter plates (Nunc, Roskilde, Denmark) overnight at 4°C in buffer containing 50 mM $NaHCO_3$ pH 9.8. Plates were then blocked with 2% Bovine serum albumin (BSA) in binding-buffer (20 mM Tris-HCl, 154 mM NaCl, 5 mM $CaCl_2$, 2 mM $MgCl_2$ and 0.05% Tween-20, pH 7.4) and 5 µg/ml FVIII was added for 1 h at 37°C. Recombinant mannose receptor-Fc chimera consisting of non-binding MR CTLD1-3- or mannose-specific MR CTLD4-7 carbohydrate recognition residues, recombinant human DC-SIGN-Fc or LRP1 Cluster II-Fc chimeras (R&D systems, Minneapolis, USA) were incubated with a concentration range up to 10 µg/ml on the plate. For competition assays, 5 µg/ml of Fc-chimera was pre-incubated with increasing concentrations (0-100 µg/ml) of mannan, RAP, α-2-macroglobulin, KM33 Fab_2 for 30 minutes at 37°C in binding buffer before incubation on FVIII-coated plates. Bound Fc fusion proteins were subsequently quantified by adding isotype specific anti-human IgG1-HRP in 50 mM Tris-HCl, 1M NaCl and 0.2% Tween-20, pH 7.4. Values are expressed as optical densities measured at 540 nm with the subtraction of 450 nm.

Administration of FVIII, KM33, VK34 and control IgG1 in Hemophilic E17 KO mice

Recombinant human FVIII was diluted to 10 µg/ml in sterile PBS and a dose of 100 µl (1 µg) was weekly administered intravenously (i.v.) in E17 KO mice (groups of 8) for either three or five weeks. In experiments where KM33, VK34 and control IgG1 (Cγ1k) were used, 1 mg of purified antibody in 500 µl sterile PBS was pre-administered intraperitoneally (i.p.), which was followed by weekly injections of 1 µg/ml FVIII. After 3 or 5 weeks, animals were sacrificed and blood samples were collected. In accordance with Dutch Laws on Animal Experimentation, research proposals involving animal handling were approved by the local Animal Experimental Committee.

Results

FVIII is internalized by immature MDDCs

To examine the uptake of FVIII by antigen-presenting cells, we used monocyte-derived dendritic cells (MDDCs) isolated from healthy subjects. Incubation of increasing concentrations of FVIII with MDDCs resulted in dose dependent FVIII endocytosis (Figure 1A). To investigate the ratio of internalized versus cell surface bound FVIII, MDDCs were incubated with FITC conjugated anti-FVIII antibody CLB-CAg117 in presence or absence of 0.05% saponin following incubation with FVIII. A large shift in fluorescent signal was observed following permeabilization with saponin indicating that the majority of FVIII is endocytosed by MDDCs (Figure 1B). To quantify FVIII uptake, we used a light chain-specific FVIII ELISA.[26] A dose-dependent increase of internalized FVIII was observed (Figure 1C). We also measured the amount of internalized FVIII heavy chain. As expected equal amounts of FVIII heavy and light chain were internalized suggesting that heterodimeric FVIII is endocytosed by MDDCs (Figure 1D). Our data show effective FVIII internalization by immature MDDCs.

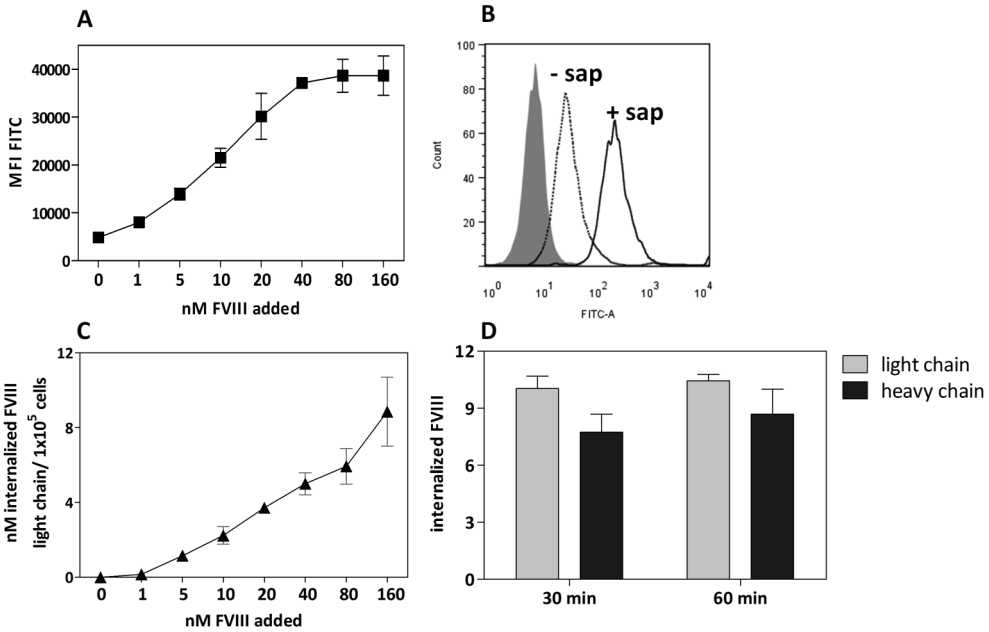

Figure 1. FVIII uptake by MDDCs

A: FVIII (0-160 nM) was incubated with MDDCs.Cells were analyzed by FACS. **B:** Cell surface bound versus internalized FVIII was compared in absence or presence of saponin. **C:** Internalized FVIII was quantified from cell lysate by ELISA. **D:** Uptake of FVIII light- and heavy chain was evaluated from cell lysates by ELISA. Results represent 4 individual experiments.

FVIII uptake is mediated by the C1 domain

To address the structural requirements for FVIII uptake, we employed two monoclonal antibodies with known specificity: antibody VK34 is directed against the A2 domain (484-508), whereas antibody KM33 targets the C1 domain.[9,22,27,28] Previously we have shown that these antibodies block the interaction of FVIII with LRP.[23,24] We hypothesized that these antibodies interfere with the uptake of FVIII by MDDCs. To test this hypothesis 80 nM KM33 or VK34 were preincubated with 10 nM FVIII and then administered for 30 minutes to MDDCs. Addition of KM33 completely abrogated FVIII uptake (Figure 2A), however FVIII endocytosis was not impaired in the presence of the anti-A2 antibody VK34 (Figure 2A). Uptake of Lucifer yellow (LY) by macropinocytosis was not affected by the addition of either KM33 or VK34 (Figure 2A). To further determine their inhibitory capacity, increasing concentrations of both antibodies KM33 and VK34 (1-160 nM) were added to 10 nM FVIII. Following the addition 40 nM KM33, we observed 50% decrease in FVIII internalization (Figure 2B). To accomplish complete blockade, 80-160 nM of KM33 was required. Similar amounts of VK34 did not influence the uptake of FVIII (Figure 2B). Assessment of internalized FVIII by ELISA confirmed the dose-dependent inhibition of FVIII uptake by KM33 (Figure 2C). Uptake of Lucifer yellow by equivalent ranges (1-160 nM) of KM33 and VK34 was not influenced (Figure 2D). These results emphasize the essential role of C1 domain in the uptake of FVIII by MDDCs.

FVIII uptake is independent from LRP, MR and DC-SIGN

Immature dendritic cells express various endocytic receptors including LRP and C-type lectin family member mannose receptor (MR, CD206). Both of these receptors have been demonstrated to associate with FVIII.[16,21] To investigate the involvement of LRP in FVIII uptake, MDDCs were preincubated with LDL receptor family member antagonist receptor associated protein (RAP) and the LRP-specific ligand α2-macroglobulin (α2m). Addition of α2m or RAP did not reduce FVIII uptake (Figure 3A). This indicates that FVIII endocytosis by MDDCs is not dependent on LRP or other LDL-receptor family members. We subsequently examined whether C-type lectins mediate FVIII uptake. Addition of increasing concentrations of mannan did not affect the uptake of FVIII by MDDCs (Figure 3A). In agreement with this finding we found that blocking antibody directed against DC-SIGN did not interfere with FVIII uptake (Figure 3A). We further tested these findings using siRNA mediated knockdown of LRP, MR and DC-SIGN. Strongly reduced expression of these receptors was observed in MDDCs treated with LRP, MR and DC-SIGN siRNAs (Figure 3B). Knockdown of these receptors was further confirmed using selective ligands. Uptake of α2m-FITC, SO_4-3-Gal and Btri was significantly lower in MDDCs treated with siRNA targeting LRP, MR and DC-SIGN, respectively (Figure 3C). FVIII uptake in MDDCs transfected with siRNA targeting LRP was similar to that detected in non-targeting (scramble) siRNA transfected cells (Figure 3D). We found similar results with MDDCs transfected with MR or DC-SIGN siRNAs, we did not observe any reduction in FVIII uptake. Altoghether, our data indicate that LRP, MR and DC-SIGN are not essential for FVIII uptake.

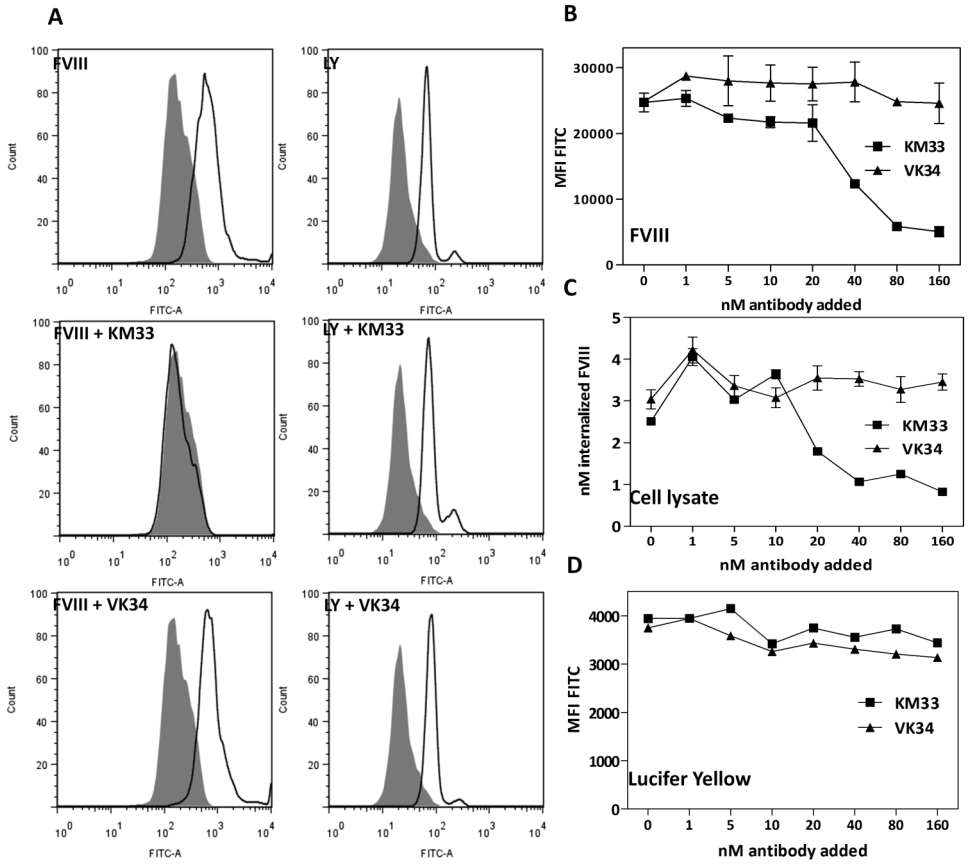

Figure 2. FVIII uptake is mediated by the C1 domain

A: 10 nM FVIII or 250 µg/ml Lucifer yellow (LY) were preincubated with 80 nM KM33 or VK34.**B, C:** KM33 or VK34 (0-160 nM) were added to 10 nM FVIII.Uptake was analyzed by FACS (B) or ELISA (C)**. D:** 250 µg/ml LY was preincubated with KM33 and VK34 before addition to MDDCs. Graphs represent data of 3 independent experiments.

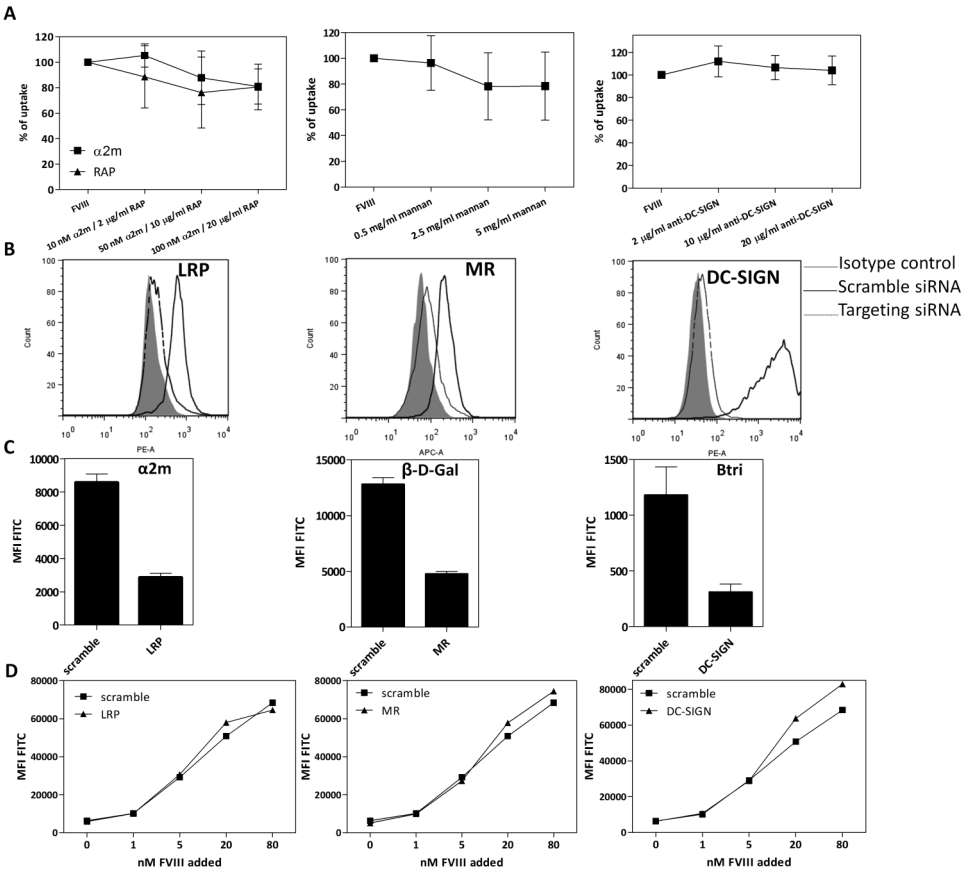

Figure 3. Uptake of FVIII by MDDCs is LRP-, MR-, and DC-SIGN independent
A: MDDCs were preincubated with α2m, RAP, mannan or anti-DC-SIGN before 40 nM FVIII was added to the cells.**B:** Receptor expression 72 hours after siRNA transfection was measured by FACS.**C:** 20 μg/ml α2m-FITC, SO_4-3-Gal or Btri was used to monitor the endocytosis through targeted receptors. **D:** Internalized FVIII (0-80 nM FVIII) was quantified by FACS Data represent 3 independent experiments.

***In vitro* binding of FVIII to LRP1 Cluster II-Fc, MR CTLD4-7-Fc and DC-SIGN-Fc chimeras**
Our findings suggest that LRP, MR and DC-SIGN are not involved in the uptake of FVIII. Previously however, it has been shown that FVIII can interact with LRP[20] and the extracellular domain of MR.[16] We re-explored this issue by addressing the capacity of FVIII to bind to cluster II ligand binding domain of LRP1-Fc fusion protein and the mannose-binding recognition domains of MR CTLD4-7-Fc chimera.[29] Additionally, we tested the binding of FVIII to DC-SIGN-Fc. When increasing concentrations of LRP1 Cluster II-Fc were added to immobilized

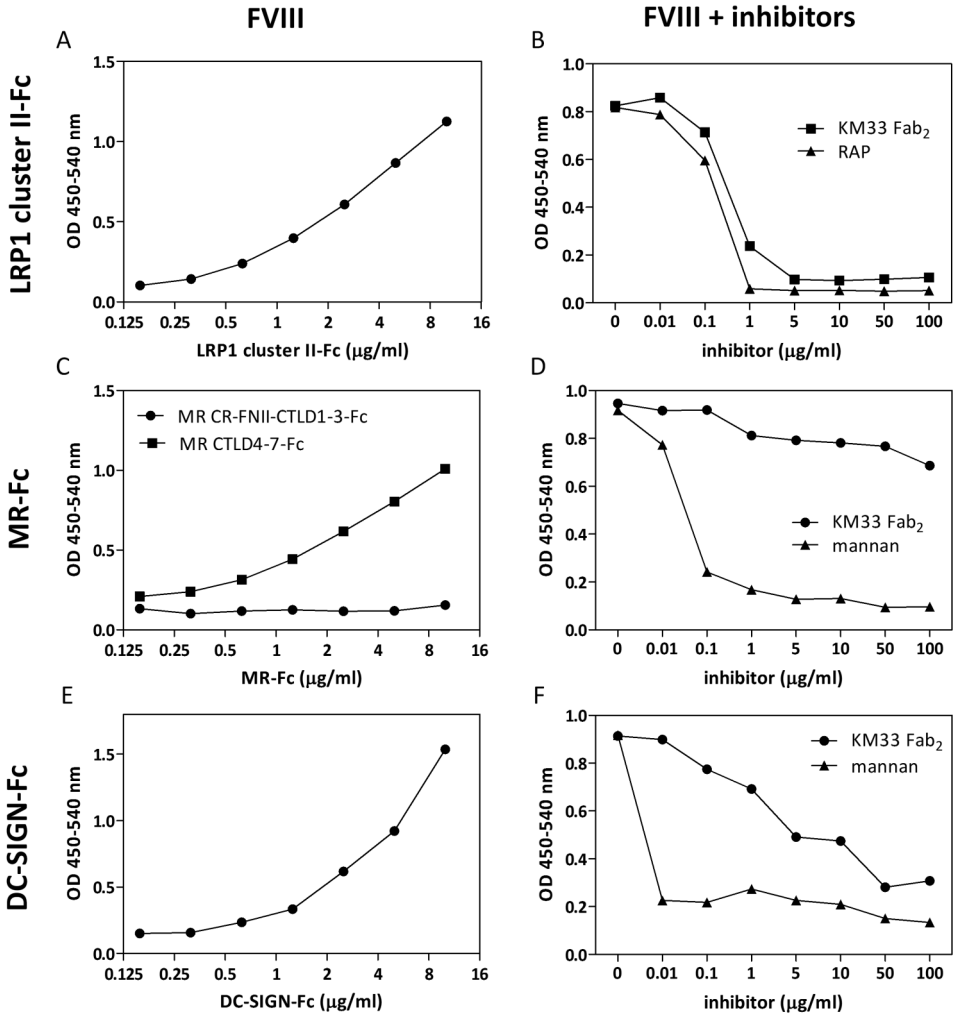

Figure 4. KM33 blocks binding of FVIII to LRP and DC-SIGN but not MR

A, C, E: LRP1 Cluster II-Fc (A), MR CTLD1-3-Fc or CTLD4-7-Fc (C), or DC-SIGN-Fc (E) (0-10 µg/ml) were added to immobilized FVIII. **B, D, F:** LRP1 Cluster II-Fc (B), MR-Fc CTLD4-7-Fc (D) or DC-SIGN-Fc (F) were pre-incubated with KM33 Fab_2 (0-100 µg/ml)**.B, D, F:** RAP (B) and mannan (D, F) were used as positive controls. Data represent 2 experiments.

FVIII on the plates, dose-dependent binding was observed (Figure 4A). Then, we addressed whether KM33 blocks binding of LRP1 Cluster II-Fc to FVIII. KM33 Fab_2 fragment inhibited LRP1 Cluster II-Fc binding to FVIII (Figure 4B). As expected, addition of Receptor-associated protein (RAP) efficiently blocked the interaction. As expected, α2m failed to compete with FVI-II binding to LRP1 Cluster II-Fc (data not shown), which is compatible with previous reports showing that combination of both cluster I and II of LRP1 is required for α2m recognition.[30] Next, we studied FVIII binding to mannose-binding carbohydrate recognition domains MR CTLD4-7-Fc and non-binding residues MR CTLD1-3-Fc. MR CTLD4-7-Fc were efficiently binding to FVIII and no interaction with MR CTLD1-3-Fc was detected, which is in agreement with previous findings[16] (Figure 4C). Following its preincubation with MR CTLD4-7-Fc, mannan completely abolished the binding to immobilized FVIII (Figure 4D), which indicates that FVIII interacts with the lectin binding domain of MR *in vitro* in a mannan-dependent manner.

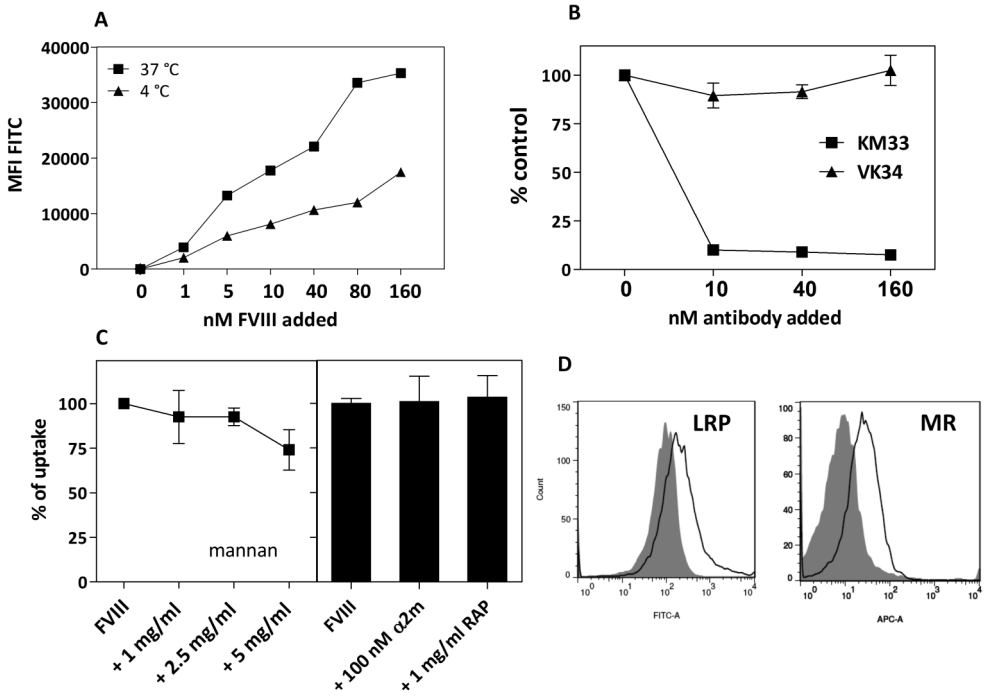

Figure 5. Uptake of FVIII by BMDCs

A: FVIII (0-160 nM) was incubated with BMDCs. **B:** 0-160 nM KM33 or VK34 was preincubated with 20 nM FVIII.**C:** Prior to endocytosis of 10 nM FVIII, BMDCs were pre-incubated with 1, 2.5 or 5 mg/ml mannan, 100 nM α2m or 1 mg/ml RAP **D:** Expression of LRP and MR were determined by FACS. Results represent 3 individual experiments.

When Fab_2 fragments of KM33 were added to FVIII, the interaction between MR CTLD4-Fc and FVIII was not affected (Figure 4D). We also assessed binding of FVIII to DC-SIGN-Fc. We observed dose-dependent binding of DC-SIGN-Fc to FVIII (Figure 4E), which was effectively inhibited by the addition of low concentrations of mannan (Figure 4F). Fab_2 fragments of KM33 at high concentrations (>5 μg/ml) significantly interfered with the interaction between DC-SIGN-Fc and FVIII (Figure 4F). Similar results were obtained when we replaced Fab_2 fragments of KM33 with KM33 IgG4. In Figure 2 we have shown that KM33 completely abolishes FVIII uptake by MDDCs. The ability of KM33 to compete with binding of FVIII to LRP and DC-SIGN but not with MR suggests that the binding of FVIII to LRP and DC-SIGN but not to MR is dependent on residues in the C1 domain of FVIII that are also involved in binding of KM33.

Uptake of FVIII by BMDCs

We subsequently studied FVIII endocytosis by murine bone marrow-derived DCs (BMDCs). FVIII was effectively endocytosed at 37°C and FVIII binding to the cell surface (at 4°C) was also observed (Figure 5A). To confirm the inhibitory effect of KM33 on the uptake of FVIII by BMDCs, increasing concentrations of KM33 were pre-incubated with FVIII prior to uptake. As control, VK34 was used. FVIII endocytosis was completely blocked by KM33, whereas VK34 did not influence its uptake (Figure 5B). Both MR and LRP are expressed on BMDCs (Figure 5D). To study the contribution of MR to FVIII uptake, we first pre-incubated increasing concentrations of mannan with BMDCs prior to addition of FVIII. Similarly to the results with MDDCs (Figure 3A), FVIII uptake was only partially affected by mannan (Figure 5C). To analyze the involvement of LRP in FVIII endocytosis by BMDCs, we used α2m and RAP (Figure 5B). We observed no reduction of internalized FVIII, which demonstrates that LRP is not essential for FVIII uptake by BMDCs. Our results demonstrate that in BMDCs, similarly to MDDCs, FVIII endocytosis is mediated by the C1 domain of FVIII, and although both MR and LRP are expressed on BMDCs, FVIII uptake is not dependent on these receptors.

KM33 modulates immune responses against FVIII in a murine model for hemophilia A

Our data suggest that the C1 residues that are targeted by KM33 are crucial for FVIII uptake by MDDCs and BMDCs. This finding prompted us to investigate whether KM33 has any modulatory effect on antibody formation *in vivo*. To test this, we used hemophilic E17 KO mice. Prior to weekly FVIII administrations, the animals received a single injection of KM33, VK34 or control IgG. After 3 weeks, the control IgG and the VK34 group's plasma contained significant amount of anti-FVIII antibodies. The response in the VK34 group was rather heterogeneous but significantly higher when compared to KM33 (Mann-Whitney U test; $p<0.05$). These results indicate that pre-injection of KM33 prevented antibody production against FVIII (Figure 6A). After 5 weeks, however, this effect disappeared (Figure 6B) due to the fact that KM33 was cleared from the circulation after day 12 (Figure 6D). Assessment of FVIII inhibitory capacity

of plasma samples using the Bethesda assay yielded similar results (Figure 6C). These data suggest that C1 domain directs the uptake of FVIII by antigen-presenting cells and is therefore essential for generation of immune responses against FVIII *in vivo*.

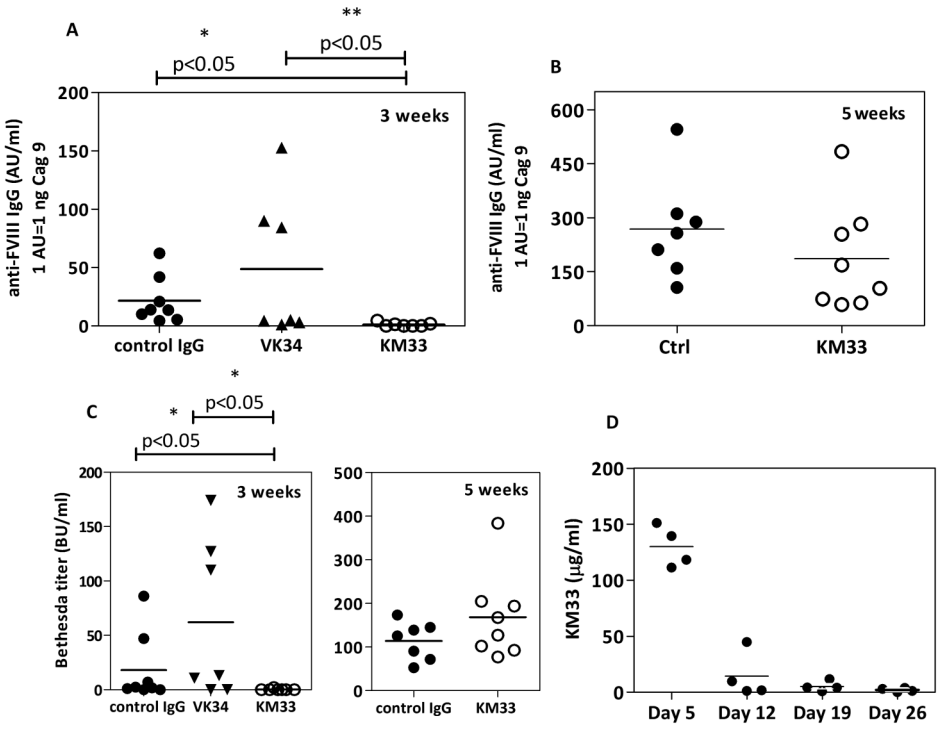

Figure 6. KM33 modulates immune responses against FVIII in a murine model for hemophilia A.

A, B: Hemophilic E-17 mice were pre-administered with 1 mg control antibody or KM33, followed by 3 (A) or 5 (B) weekly FVIII injection .Anti-FVIII antibody titers from the collected plasma samples were determined. **C:** Inhibitory capacity of FVIII antibodies were measured by Bethesda assay. **D:** Presence of KM33 in blood was monitored. Data was analyzed using non-parametric Mann-Whitney U-test.

Discussion

Immunogenicity of biopharmaceuticals delivers serious, sometimes life-threatening complications.[31] FVIII replacement therapy is no exception, since inhibitory antibody formation occurs in approximately 20% of the patients with severe hemophilia A.[32] Both genetic risk factors and treatment-related parameters contribute to the etiology of inhibitor formation in hemophilia A.[32,33] Induction of immune responses to FVIII subsequent to its intravenous infusion is most likely initiated in the spleen.[13,34] Marginal zone metallophilic macrophages as well as dendritic cells that are present underneath the marginal sinus endothelial cells contribute to sampling of antigens from blood.[35] Subsequent uptake and processing of antigen results in presentation of peptides on MHC molecules allowing for instruction of naïve T cells in the T-cell zone of the white pulp.[35] In this study we employed monocyte-derived dendritic cells (MDDCs) to explore determinants on FVIII and MDDCs that are involved in the uptake of FVIII. We found that FVIII is internalized by a mechanism that is inhibited by antibody KM33 that targets the C1 domain of FVIII. However, antibody VK34 that is directed towards the A2 domain of FVIII failed to influence FVIII uptake. These data suggest that residues within the C1 domains are essential for the uptake of FVIII by MDDCs. Previously, we have shown that KM33 prohibits the binding of FVIII to phosphatidyl-L-serine (Ptd-L-Ser).[28] This raises the possibility that Ptd-L-Ser-exposure dictates endocytosis of FVIII. However, staining with FITC-labelled annexin V revealed that immature MDDCs did not express appreciable amounts of Ptd-L-Ser under our experimental conditions (data not shown). FVIII can interact with surface receptors like LRP, asialoglycoprotein receptors as well as MR.[16,20,21,36] Both LRP and MR are expressed on human MDDCs, nevertheless our findings suggest that these receptors do not contribute to FVIII endocytosis. Several explanations can be forwarded to explain this apparent discrepancy. One can argue that the ligand binding domains of LRP and MR are not sufficiently exposed to allow for internalization of FVIII. Binding of FVIII to LRP is mediated by repetitive complement-type repeats which are arranged in four clusters.[37] Complement-type repeats CR5-8 and CR24-26 in cluster II and IV can support binding to FVIII when present as isolated fragments.

One cannot exclude that these interactive sites are cryptic in full length LRP. This could potentially explain the lack of involvement of LRP in endocytosis of FVIII in MDDCs and BM-DCs observed in this and a previous study.[38] Mannosylation of Asn^{239} and Asn^{2118} of FVIII has been implicated in the internalization of FVIII by DCs through binding to MR.[16] In accordance with Dasgupta and co-workers we show that FVIII can interact with the lectin binding domain of MR *in vitro* (see Figure 4) in a mannan-sensitive manner. However, KM33 does not inhibit binding of FVIII to MR. In agreement with these findings, we did not observe a decline in FVIII uptake in the presence of mannan (Figure 3), which excludes a prominent role for C-type lectins in FVIII endocytosis. Furthermore, using a more specific approach, siRNA-mediated knockdown of MR expression did not result in decline in FVIII uptake (Figure 3B). Additionally, simultaneous siRNA mediated knockdown of MR, LRP and DC-SIGN also did

not abrogate FVIII uptake (Online Repository Materials Figure 2). Taken together, these results indicate that MR is not involved in FVIII uptake in human MDDCs. Staining of MR in macrophages and DCs reveal that a major part of this protein resides in early endosomes.[39] This finding is compatible with the constitutive clathrin-mediated endocytosis of MR.[40] As such MR may not be able to compete for endocytosis of FVIII by other surface components. FVIII uptake followed by its presentation on MHC class II molecules leads to antibody production in hemophilic E17 KO mice. Here, we show that blockade of uptake by KM33 prevents immune responses against FVIII. However, our data indicate that this effect does not result in long-term tolerance to FVIII; after KM33 is removed from the circulation, its inhibitory effect disappears. Taken together, the inhibitory effect of KM33 *in vitro* and *in vivo* suggest that this antibody targets an epitope of FVIII that is essential for its uptake. Here, we show that KM33 interferes with the binding of FVIII to LRP and DC-SIGN and not with MR, although these receptors are not involved in FVIII *in vitro* uptake perhaps due to their low-affinity binding to FVIII. Further studies are needed to address the role of these receptors *in vivo*. Alternatively, another possible explanation for the effect of the KM33 antibody addition is that the antibody KM33 stabilized FVIII in an alternative conformation that prevented its recognition and uptake by DCs. Nevertheless, our findings raise the possibility that a yet unidentified receptor plays a critical role in FVIII uptake by DCs by binding to an interactive surface in the C1 domain of FVIII that is also targeted by KM33. Our *in vivo* findings suggest that this interactive surface in the C1 domain is critical for initiation of immune responses to FVIII.

References

1. Mannucci PM, Tuddenham EG. The hemophilias--from royal genes to gene therapy. *N Engl J Med*. 2001;344(23):1773-1779.
2. Kempton CL, White GC, 2nd. How we treat a hemophilia A patient with a factor VIII inhibitor. *Blood*. 2009;113(1):11-17.
3. Fijnvandraat K, Bril WS, Voorberg J. Immunobiology of inhibitor development in hemophilia A. *SeminThrombHemost*. 2003;29(1):61-68.
4. Lollar P. Pathogenic antibodies to coagulation factors. Part one: factor VIII and factor IX. *J Thromb Haemost*. 2004;2(7):1082-1095.
5. Dimichele D. Immune tolerance therapy for factor VIII inhibitors: moving from empiricism to an evidence-based approach. *J Thromb Haemost*. 2007;5 Suppl 1:143-150.
6. Reding MT, Wu H, Krampf M, et al. Sensitization of CD4+ T cells to coagulation factor VIII: response in congenital and acquired hemophilia patients and in healthy subjects. *Thromb Haemost*. 2000;84(4):643-652.
7. van Helden PM, van den Berg HM, Gouw SC, et al. IgG subclasses of anti-FVIII antibodies during immune tolerance induction in patients with hemophilia A. *Br J Haematol*. 2008;142(4):644-652.
8. Jacquemin MG, Desqueper BG, Benhida A, et al. Mechanism and kinetics of factor VIII inactivation: study with an IgG4 monoclonal antibody derived from a hemophilia A patient with inhibitor. *Blood*. 1998;92(2):496-506.
9. van den Brink EN, Turenhout EA, Bank CM, Fijnvandraat K, Peters M, Voorberg J. Molecular analysis of human anti-factor VIII antibodies by V gene phage display identifies a new epitope in the acidic region following the A2 domain. *Blood*. 2000;96(2):540-545.
10. Rajewsky K. Clonal selection and learning in the antibody system. *Nature*. 1996;381(6585):751-758.
11. Lacroix-Desmazes S, Navarrete AM, Andre S, Bayry J, Kaveri SV, Dasgupta S. Dynamics of factor VIII interactions determine its immunologic fate in hemophilia A. *Blood*. 2008;112(2):240-249.
12. Shortman K, Liu YJ. Mouse and human dendritic cell subtypes. *Nat Rev Immunol*. 2002;2(3):151-161.
13. Navarrete A, Dasgupta S, Delignat S, et al. Splenic marginal zone antigen presenting cells are critical for the primary allo-immune response to therapeutic factor VIII in hemophilia A. *J Thromb Haemost*. 2009;7(11):1816-1823.
14. Pfistershammer K, Stockl J, Siekmann J, Turecek PL, Schwarz HP, Reipert BM. Recombinant factor VIII and factor VIII-von Willebrand factor complex do not present danger signals for human dendritic cells. *Thromb Haemost*. 2006;96(3):309-316.
15. Qadura M, Othman M, Waters B, et al. Reduction of the immune response to factor VIII mediated through tolerogenic factor VIII presentation by immature dendritic cells. *J Thromb Haemost*. 2008;6(12):2095-2104.
16. Dasgupta S, Navarrete AM, Bayry J, et al. A role for exposed mannosylations in presentation of human therapeutic self-proteins to CD4+ T lymphocytes. *Proc Natl Acad Sci U S A*. 2007;104(21):8965-8970.
17. Medzihradszky KF, Besman MJ, Burlingame AL. Structural characterization of site-specific N-glycosylation of recombinant human factor VIII by reversed-phase high-performance liquid chromatography-electrospray ionization mass spectrometry. *Anal Chem*. 1997;69(19):3986-3994.

18. Garcia-Vallejo JJ, van Kooyk Y. Endogenous ligands for C-type lectin receptors: the true regulators of immune homeostasis. *Immunol Rev*. 2009;230(1):22-37.
19. Bovenschen N, Herz J, Grimbergen JM, et al. Elevated plasma factor VIII in a mouse model of low-density lipoprotein receptor-related protein deficiency. *Blood*. 2003;101(10):3933-3939.
20. Lenting PJ, Neels JG, van den Berg BM, et al. The light chain of factor VIII comprises a binding site for low density lipoprotein receptor-related protein. *J Biol Chem*. 1999;274(34):23734-23739.
21. Saenko EL, Yakhyaev AV, Mikhailenko I, Strickland DK, Sarafanov AG. Role of the low density lipoprotein-related protein receptor in mediation of factor VIII catabolism. *J Biol Chem*. 1999;274(53):37685-37692.
22. van den Brink EN, Turenhout EA, Bovenschen N, et al. Multiple VH genes are used to assemble human antibodies directed toward the A3-C1 domains of factor VIII. *Blood*. 2001;97(4):966-972.
23. Bovenschen N, Boertjes RC, van Stempvoort G, et al. Low density lipoprotein receptor-related protein and factor IXa share structural requirements for binding to the A3 domain of coagulation factor VIII. *J Biol Chem*. 2003;278(11):9370-9377.
24. Bovenschen N, van Stempvoort G, Voorberg J, Mertens K, Meijer AB. Proteolytic cleavage of factor VIII heavy chain is required to expose the binding-site for low-density lipoprotein receptor-related protein within the A2 domain. *J Thromb Haemost*. 2006;4(7):1487-1493.
25. van Haren SD, Herczenik E, ten Brinke A, Mertens K, Voorberg J, Meijer AB. HLA-DR-presented peptide repertoires derived from human monocyte-derived dendritic cells pulsed with blood coagulation factor VIII. *Mol Cell Proteomics*. 2011;10(6):M110 002246.
26. van den Brink EN, Bril WS, Turenhout EA, et al. Two classes of germline genes both derived from the V(H)1 family direct the formation of human antibodies that recognize distinct antigenic sites in the C2 domain of factor VIII. *Blood*. 2002;99(8):2828-2834.
27. Amini-Nekoo A, Futers TS, Moia M, Mannucci PM, Grant PJ, Ariens RA. Analysis of the tissue factor pathway inhibitor gene and antigen levels in relation to venous thrombosis. *Br J Haematol*. 2001;113(2):537-543.
28. Meems H, Meijer AB, Cullinan DB, Mertens K, Gilbert GE. Factor VIII C1 domain residues Lys 2092 and Phe 2093 contribute to membrane binding and cofactor activity. *Blood*. 2009.
29. Martinez-Pomares L, Wienke D, Stillion R, et al. Carbohydrate-independent recognition of collagens by the macrophage mannose receptor. *Eur J Immunol*. 2006;36(5):1074-1082. 30. Mikhailenko I, Battey FD, Migliorini M, et al. Recognition of alpha 2-macroglobulin by the low density lipoprotein receptor-related protein requires the cooperation of two ligand binding cluster regions. *J Biol Chem*. 2001;276(42):39484-39491.
31. Kessler M, Goldsmith D, Schellekens H. Immunogenicity of biopharmaceuticals. *Nephrol Dial Transplant*. 2006;21 Suppl 5:v9-12.
32. Gouw SC, van der Bom JG, Marijke van den Berg H. Treatment-related risk factors of inhibitor development in previously untreated patients with hemophilia A: the CANAL cohort study. *Blood*. 2007;109(11):4648-4654.
33. Pavlova A, Brondke H, Musebeck J, Pollmann H, Srivastava A, Oldenburg J. Molecular mechanisms underlying hemophilia A phenotype in seven females. *J Thromb Haemost*. 2009;7(6):976-982.
34. Waters B, Qadura M, Burnett E, et al. Anti-CD3 prevents factor VIII inhibitor development in hemophilia A mice by a regulatory CD4+CD25+-dependent mechanism and by shifting cytokine production to favor a Th1 response. *Blood*. 2009;113(1):193-203.

35. Mebius RE, Kraal G. Structure and function of the spleen. *Nat Rev Immunol*. 2005;5(8):606-616.
36. Bovenschen N, Mertens K, Hu L, Havekes LM, van Vlijmen BJ. LDL receptor cooperates with LDL receptor-related protein in regulating plasma levels of coagulation factor VIII in vivo. *Blood*. 2005;106(3):906-912.
37. Meijer AB, Rohlena J, van der Zwaan C, et al. Functional duplication of ligand-binding domains within low-density lipoprotein receptor-related protein for interaction with receptor associated protein, alpha2-macroglobulin, factor IXa and factor VIII. *Biochim Biophys Acta*. 2007;1774(6):714-722.
38. Dasgupta S, Navarrete AM, Andre S, et al. Factor VIII bypasses CD91/LRP for endocytosis by dendritic cells leading to T-cell activation. *Haematologica*. 2008;93(1):83-89.
39. Burgdorf S, Kurts C. Endocytosis mechanisms and the cell biology of antigen presentation. *Curr Opin Immunol*. 2008;20(1):89-95.
40. Stahl P, Schlesinger PH, Sigardson E, Rodman JS, Lee YC. Receptor-mediated pinocytosis of mannose glycoconjugates by macrophages: characterization and evidence for receptor recycling. *Cell*. 1980;19(1):207-215.

Chapter 4

Mutations within the C1 domain of FVIII reduce its immunogenicity *in vitro* and *in vivo*.

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Submitted for publication

Abstract

The unwanted formation of FVIII-neutralizing antibodies in patients with hemophilia A is initiated by the endocytosis of FVIII by professional antigen-presenting cells. Endocytosis of FVIII by human monocyte-derived dendritic cells can be significantly blocked by monoclonal anti-FVIII antibody KM33. In this study, we constructed a FVIII variant with alanine substitutions at positions 2090, 2092 and 2093, which completely diminishes binding to KM33. Similar to the effect of KM33, this variant is not internalized by murine bone marrow-derived dendritic cells. We also investigated the *in vivo* ability of this variant to induce inhibitors in haemophilic mice. E17 KO mice develop inhibitors in response to multiple FVIII injections. We show that E17 KO mice treated with the FVIII variant have significantly lower anti-FVIII antibody titers than mice treated with wild-type FVIII. Alanine substitutions at positions 2090, 2092 and 2093 result in a FVIII molecule that is significantly less immunogenic than wild-type FVIII both *in vitro and in vivo*, due to its diminished internalization by various antigen-presenting cell types, but has pro-coagulant activity similar to wild-type FVIII. These properties make this FVIII variant a therapeutically interesting molecule for improved treatment of hemophilia A patients.

Introduction

The X-chromosome-linked bleeding disorder hemophilia A is caused by the absence or dysfunction of clotting factor VIII (FVIII), which can be corrected by regular intravenous administration of FVIII. Development of antibodies against FVIII (referred as inhibitors) that can hinder its function is a severe complication in the treatment of hemophilia A. The presence of inhibitors significantly changes the treatment outcome, since regular prophylactic administration of FVIII no longer has an effect. Patients with inhibitors can be treated with bypassing agents including activated Factor VII (FVII). However, since prophylaxis is the preferred treatment, the strategy in most cases is to try to eradicate the FVIII inhibitors. In such cases a treatment called immune tolerance induction (ITI) is introduced, which consists of more frequent administrations of FVIII and a higher dose.[1] This generally results in a reduction of the inhibitor titer.[2-4] However, ITI is a very expensive therapy and unfortunately not always successful. Moreover, successful tolerance induction can in some cases take up to 2 years.[5] An interesting alternative is to change the treatment regimen in such a way that the development of inhibitors can be prevented. A thorough understanding of how inhibitor development takes place is necessary in order to be able to prevent inhibitor formation.

What is known is that inhibitor formation is a T-cell dependent process in both hemophilic mice[6-8] and hemophilia A patients.[9-12] Activation of FVIII-specific T cells is preceded by the uptake of FVIII by antigen-presenting cells (APCs) and the subsequent presentation of FVIII peptides on MHC class II molecules on the surface of these APCs.[13] The interactions between APCs and $CD4^+$ T cells in the development of inhibitors have been investigated in several studies using hemophilic mice with a functional disruption of the FVIII gene.[14] Van Schooten and coworkers show that administration of human FVIII resulted in clearance of human FVIII in the spleen and liver by $CD68^+$ macrophages in von Willebrand Factor (VWF)-deficient mice.[15] In a publication by Navarrete et al., the uptake of FVIII was attributed more specifically to splenic marginal zone metallophilic macrophages in FVIII-deficient mice.[16] This uptake is essential for the formation of inhibitors, since *in vivo* depletion of these cells resulted in a reduction of inhibitor formation. Another important requirement for APCs ability to activate FVIII-specific $CD4^+$ T cells is to provide the T cell with co-stimulation. Interfering with the activation of T cells by APCs has proven to be a successful strategy to reduce inhibitor formation. The formation of anti-FVIII antibodies in mice was reduced by blocking the interaction between co-stimulatory molecules B7 and CD28.[6] Lentiviral administration of the FVIII gene into neonatal mice, which are in an early stage of life in which central tolerance to both self and exogenously introduced antigens is established, even leads to the induction of long-lasting tolerance for FVIII.[17]

In this study, instead of blocking the activation of FVIII-specific T cells, we explored whether it is feasible to reduce the recognition and endocytosis of FVIII by APCs. Previously, we have shown that monoclonal antibody KM33, directed against the C1 domain of FVIII, was able to significantly block the endocytosis of FVIII by human monocyte-derived DCs as well

as murine bone marrow-derived DCs.[18] Recently, the C1 domain residues lysine 2092 and phenylalanine 2093 were identified as amino acids that contribute significantly to the binding to KM33.[19] In this study we show that mutating those residues to alanine results in a FVIII variant which shows reduced endocytosis by murine antigen-presenting cells. Moreover, an additional substitution of an arginine at position 2090 to an alanine displays pronounced reduction in immunogenicity both *in vitro* as well as *in vivo*, while retaining the same level of procoagulant activity. Our findings show the potential of reducing FVIII immunogenicity in hemophilia A by selective modulation of its endocytosis by antigen-presenting cells.

Methods

Materials

For culturing bone-marrow derived murine DCs (BMDCs), mouse recombinant GM-CSF was purchased (R&D System, Minneapolis, MN, USA). Penicillin/streptomycin, DMEM/F12, RPMI-1640 and serum-free X-VIVO 15 medium were from Lonza (Walkersville, MD, USA). Fetal calf serum was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Cell factories (6320 cm^2), culture flasks and 96-well microtiter plates were purchased from Nunc (Roskilde, Denmark). Ultrapure methanol-free paraformaldehyde was from Polysciences (Eppelheim, Germany).

Antibodies

Anti-murine CD83-APC, anti-murine CD86, anti-murine CD11b-FITC, rat IgG isotype control conjugated with FITC, APC or biotin, streptavidin-APC were purchasd from BD Biosciences (San Jose, CA, USA); Anti-mouse CD14-biotin, anti-mouse CD45R-biotin and anti-mouse Gr-1-biotin (eBioscience); Anti-murine CD11c producing cell line (clone HB-224) was purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). Monoclonal antibodies CLB-CAg117, CLB-CAg9, CLB-CAg69 and CLB-CAg12 targeting different domains of FVIII have been described previously.[20] Recombinant KM33, VK34 and control IgG1 (Cγ1k) were prepared as described previously.[21,22] Full-length antibodies were expressed in HEK-293 cells and purified using protein A Sepharose (GE Healthcare, United Kingdom). For flow cytometry, antibody CLB-Cag117 was labeled with FITC using the FluoReporter® FITC Protein Labeling Kit (Invitrogen, Breda, the Netherlands).

Preparation of factor VIII mutants

B domain-deleted FVIII and mutants were constructed as described previously.[19,23] Arg2090Ala, Lys2092Ala and Phe2093Ala mutations were introduced by QuickChange site-directed mutagenesis. Coding regions of all constructs were verified by sequence analysis. Sequence reaction was performed using BigDye Terminator Cycle Sequencing kit (Applied Biosystems). HEK293 stable cell lines expressing recombinant proteins were prepared as described previously.[24] Cells were cultured in DMEM/F-12 medium in presence of 10% fetal calf serum. All proteins were purified by immunoaffinity chromatography using sepharose coupled with monoclonal anti-FVIII antibody VK34 as described previously.[25] Protein concentration was measured by Bradford protein assay. FVIII concentration was determined by enzyme-linked immunosorbent assay essentially as described.[26] FVIII activity was determined by a chromogenic assay according to manufacturer's instructions (Chromogenix).

Binding of FVIII to KM33

FVIII binding to KM33 was measured by sandwich ELISA and using surface plasmon resonance analysis. For ELISA assay, Nunc-Maxisorp 96-well plates were coated with KM33 IgG1 (5 µg/ml) antibody in carbonate buffer (50 mM NaHCO3 pH 9.8) overnight at 4°C. Subsequently, FVIII variants were incubated in binding buffer in a concentration range up to 1 U/ml on the plate. Bound FVIII was detected with horseradish peroxidase conjugated mAb CLB-CAg117 (targeting C2 domain of FVIII) or CLB-CAg9 (targeting A2 domain of FVIII). Values are expressed as optical densities measured at 540 nm with the subtraction of 450 nm. Surface plasmon resonance analysis was performed using a BIAcore3000 biosensor system (Biacore AB) as described previously.[19] Human monoclonal antibody EL14 was covalently coupled to the dextran surface of an activated CM5-sensor chip. Subsequently, FVIII variants were loaded on EL14. Association and dissociation of 25 mM KM33 IgG1 were performed in 20 mM Hepes, 150 mM NaCl, 5 mM CaCl2 and 0.005% Tween 20 (pH 7.4) at a flow rate of 20 µl/min for 4 minutes at 25°C. Association and dissociation curves were corrected for nonspecific binding.

Hemophilic E17 KO Mice

Hemophilic E17 KO mice, characterized by a targeted disruption of exon 17 of the FVIII gene[14,27] were backcrossed into the C57BL/6J background as described previously.[28,29] Mice used in this study were male and aged between 8 and 12 weeks at the beginning of the experiment. The genotypes of hemophilic mice were confirmed by polymerase chain reaction analysis of genomic DNA extracted from ear clippings, as described previously.[6]

Murine bone-marrow-derived DCs (BMDC) were essentially prepared as described before.[30] Briefly, bone marrow cells were isolated by flushing femurs from E17 KO mice with PBS supplemented with 2% FCS. The bone marrow suspension was incubated in Tris-NH_4Cl at room temperature for 2 minutes to lyse erythrocytes. Finally, the cells were resuspended at $1x10^6$ cells/ml containing 20 ng/ml mouse recombinant GM-CSF and cultured for 8-10 days in RPMI 1640 medium supplemented with 2.5 mM HEPES, 55 mM 2-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin, 5 mM glutamine and 10% FCS. Expression of CD11c, CD11b, CD80, CD86, B220 and Gr-1 was routinely measured on day 8-10. Cells generated by this method comprised 95% DC (MHC class II^+CD11c^+), with the remainder of the cells being predominantly granulocytes.

FVIII endocytosis by flow cytometry

Approximately $2x10^5$ of DCs were first washed once with serum-free medium and incubated with FVIII in 120 µl of serum-free medium for 30 minutes at 37°C. After FVIII uptake, cells were washed with ice-cold TBS, fixed with 1% freshly prepared paraformaldehyde and incubated with FITC-conjugated monoclonal anti-FVIII antibody CLB-CAg117 in presence or absence of 0.05% saponin in TBS containing 0.5% HSA. Mean fluorescence intensities and percentage of positive cells were determined by flow cytometry using LSRII (BD Biosciences, Uppsala, Sweden).

Administration of FVIII in Hemophilic E17 KO mice

Recombinant B-domain deleted human FVIII wild-type or mutants were diluted to 10 μg/ml in sterile PBS and a dose of 100 μl (1 μg) was administered intravenously (i.v.) in E17 KO mice (n=8) five times weekly. Endotoxin levels were verified using LAL chromogenic assay (Toxin-Sensor™, GenScript, NJ, USA). One week after last FVIII injections, animals were sacrificed and blood samples and spleens were collected for further analysis.

Anti-FVIII inhibitory antibody measurements from mouse plasma by enzyme-linked immunosorbent assay (ELISA) and Bethesda assay

FVIII (5 μg/ml) was immobilized on 96 well microtiter plates (Nunc, Roskilde, Denmark) in buffer containing 50 mM NaHCO3 pH 9.8. Plates were blocked with 2% gelatin in PBS. Mouse plasma dilutions were prepared in 50 mM Tris, 150 mM NaCl, 2% BSA, pH 7.4. Mouse monoclonal anti-FVIII antibody CLB-CAg9 was used as a standard. Anti-FVIII antibodies were detected with goat anti-mouse IgG-HRP. The concentration of anti-FVIII antibodies in murine plasma are displayed in arbitrary units (AU), where 1 AU corresponds to signal obtained from 1 μg of CLB-CAg9. To identify FVIII inhibitors, we performed a Bethesda assay with Nijmegen modification.[31] Data was analyzed using non-parametric Mann-Whitney U-test.

Analysis of anti-FVIII antibody-secreting cells by enzyme-linked immunosorbent spot assay (ELISpot)

Anti-FVIII or total antibody-secreting cells (ASC) were quantified using a modification of the Elispot method described previously.[32] Briefly, FVIII or rabbit-anti-murine IgG (Dako) was immobilized to the solid phase of polyvinylidendifluorid (PVDF)-bottom 96-well multiscreen-IP filtration plates (Millipore Corporation, San Francisco, CA). Non-specific binding sites were blocked with 10% FCS in RPMI 1640. The plates were incubated for 5 h at 37°C in a humid atmosphere (5% CO2) with serial dilutions of cell suspensions obtained from spleen of mice treated with FVIII. The viability of cells was analyzed by exclusion of trypan blue (0.4% trypan blue solution, Sigma-Aldrich, Irvine, UK). After incubation, the plates were washed three times with PBS and three times with PBS containing 0.1% Tween 20 (Merck, Hohenbrunn, Germany) to remove the cells. Horseradish-peroxidase (HRP)-labeled goat anti-mouse immunoglobulin (Dako, Glostrup, Denmark) was added to each well. For IgG-subclass-specific analyses of antibody-secreting cells, IgG-subclass-specific HRP-labeled monoclonal antibodies (Southern Biotechnologies, Birmingham, AL) were used instead. Plates were incubated overnight at 4°C. Subsequently, plates were washed three times with PBS containing 0.1% Tween 20 and an additional three times with PBS only. Spots were developed using TMB-substrate (Sanquin reagents, Amsterdam, The Netherlands). Plates were washed with tap water, air-dried and then analyzed for spots using the AELvis reader and eli.analyse software version 4.1 (A.EL.VIS GMBH, Hannover, Germany).

T-cell proliferation assay

Spleens collected after weekly injections of FVIII were pooled within the groups. Erythrocytes were removed and CD8+ cells were depleted by magnetic bead separation using beads coated with the anti-mouse CD8 antibody Lyt 1.2 (eBioscience). Remaining $CD8^-$ cells were cultured in round-bottomed 96-well plates for 72 or 96 hours in X-VIVO 15 medium supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin (all from BioWhittaker; Walkersville, Maryland) and 55 μM ß-mercaptoethanol (Sigma-Aldrich, Irvine, UK) in presence of increasing FVIII concentration (0, 0.1, 0.5 or 1 μg/ml) to generate antigen-specific T-cell proliferation or concanavalin A (1 μg/ml) to generate nonspecific proliferation. Proliferation was assayed by the addition of 1 μCi/well of [^{3}H]thymidine (ICN Pharmaceuticals, Irvine, CA) for the last 16-18 hours. The results were expressed as the stimulation index (cpm with antigen/cpm with medium alone).

Binding of FVIII to vWF

FVIII binding to VWF was measured by sandwich ELISA. Briefly, Nunc-Maxisorp 96-well plates were coated with CLB-RAg20 (2.5 μg/ml) capture antibody in carbonate buffer (50 mM NaHCO3 pH 9.8) overnight at 4°C. FVIII at concentration 1 U/ml was incubated with various concentrations of vWF for 15 minutes at 37°C. The mixtures of FVIII with vWF were subsequently placed on Rag-20 coated plates. After 2 hours of incubation at 37°C, plates were washed and bound FVIII detected with horseradish peroxidase conjugated mAb CLB-CAg117 (targeting C2 domain of FVIII). Values are expressed as optical densities measured at 540 nm with the subtraction of 450 nm.

Statistical analysis

Apart from anti-FVIII inhibitory antibody measurements, data were analyzed by Student's t test and differences were considered significant at $P < 0.05$: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Results

Anti-C1 antibody KM33 inhibits FVIII uptake by bone-marrow dendritic cells

We and others have previously shown[33] that human monocyte-derived dendritic cells are able to internalize FVIII. In agreement with previous findings, we were able to block the endocytosis of FVIII by BMDCs using C1 domain-directed monoclonal antibody KM33[19], but not by VK34, a monoclonal antibody targeting the A2 domain of FVIII (Figure 1A). To explore whether uptake of FVIII by murine antigen-presenting cells can also be inhibited by KM33, we used bone-marrow dendritic cells (BMDCs). In agreement with previous findings, C1 domain directed antibody KM33 was able to abolish the uptake of FVIII by these cells[18], whereas a control antibody targeting FVIII A2 domain – VK34, did not (Figure 1).

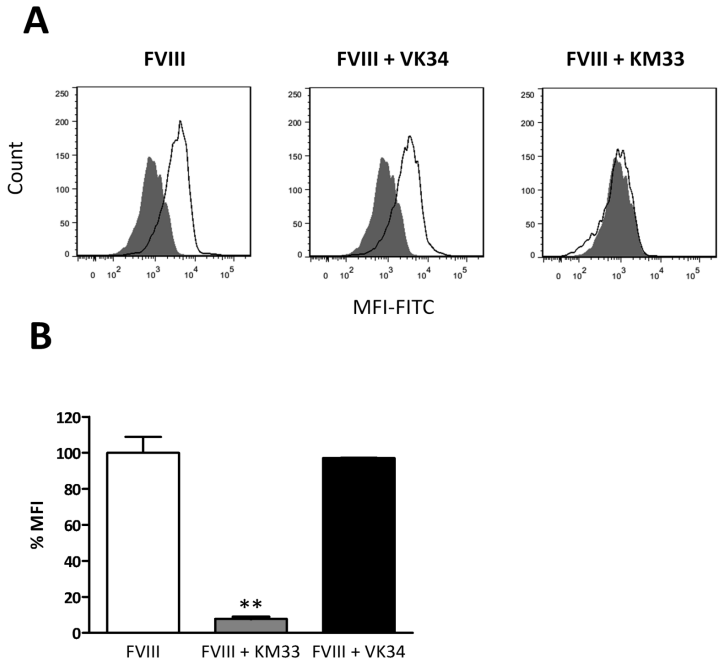

Figure 1. Endocytosis of FVIII by murine bone-marrow derived dendritic cells is mediated by the C1 domain.

10 nM of FVIII was pre-incubated with 80 nM of either C1 domain-directed antibody KM33 or A2 domain-directed antibody VK34 prior to addition to the cells. FVIII internalization was measured with CLB-CAg117-FITC. Grey filled histograms represent untreated cells. Data show mean ± SD from at least 3 independent experiments. Mean fluorescence was shown in histograms **(A)** or bar diagram **(B)**.

FVIII with alanine substitutions in positions 2090, 2092 and 2093 shows diminished binding to KM33

It was previously shown that C1 domain residues 2092 and 2093 are required for KM33 binding to FVIII[19]. However, since mutating these residues to alanines does not completely abolish KM33 binding, we hypothesize that additional C1 domain amino acids can be involved in the interaction between FVIII and KM33. To test this hypothesis, we mutated an additional positively charged amino acid present at the tip of the C1 domain (Figure 2). Arginine at position 2090 was changed to alanine. To establish whether such a mutation will have an additional effect, a FVIII variant was prepared where all three residues, 2090, 2092 and 2093, were substituted with alanines. Mutants and wild-type FVIII were expressed in HEK-293 cells and purified by immunoaffinity chromatography. To explore the binding of purified variants to KM33, we employed a solution-phase sandwich ELISA in which FVIII, bound to immobilized KM33, was detected either via an A2 or C2 domain targeting antibody (CLB-CAg9 or CLB-CAg117, respectively). As expected, FVIII 2092/2093 showed a significant reduction in ability to bind KM33 as compared to wild-type FVIII. Introduction of a single alanine substitution in position 2090 did not influence the binding, however the presence of this mutation additional to mutations in 2092/2093 showed an even more pronounced decrease in binding to KM33 (Figure 3A). To validate these findings, we performed surface plasmon resonance analysis of the FVIII-KM33 interaction. 25 nM full-length KM33 (IgG1) was passed over FVIII WT, FVIII 2092/2093 and FVIII 2090/2092/2093 as described in the Materials and Methods section. Similar to the previous observation, FVIII 2090/2092/2093 showed greater reduction of binding to KM33 as compared to the variant with substitutions only in positions 2092 and 2093 (Figure 3B). Altogether, these results confirm the importance of Arg2090, Lys2092 and Phe2093 in binding of FVIII to antibody KM33.

C1 domain mutations alter FVIII endocytosis by human antigen-presenting cells

As previously shown, KM33 abrogates the uptake of FVIII by human monocyte-derived dendritic cells, and residues 2090, 2092 and 2093 are important for binding of KM33 to FVIII. These findings suggest that this surface-exposed region in the C1 domain may be of great importance for endocytosis of FVIII by antigen presenting cells (Figure 2). To investigate this, BMDCs were incubated for 30 minutes with 15 nM FVIII wild-type or mutants, then fixed and stained with CLB-CAg117 antibody in presence of saponine, and internalized FVIII was subsequently detected by flow cytometry.

FVIII R2090A was endocytosed at a slightly reduced level as compare to FVIII wild-type (Figure 4), whereas endocytosis of FVIII 2092/2093 was more severely impaired. Uptake of FVIII 2090/2092/2093 was even more reduced when compared to FVIII-K2092A-F2093A (Figure 4). These results show that Arg2090, Lys2092 and Phe2093 contribute to the uptake of FVIII by murine bone marrow derived dendritic cells.

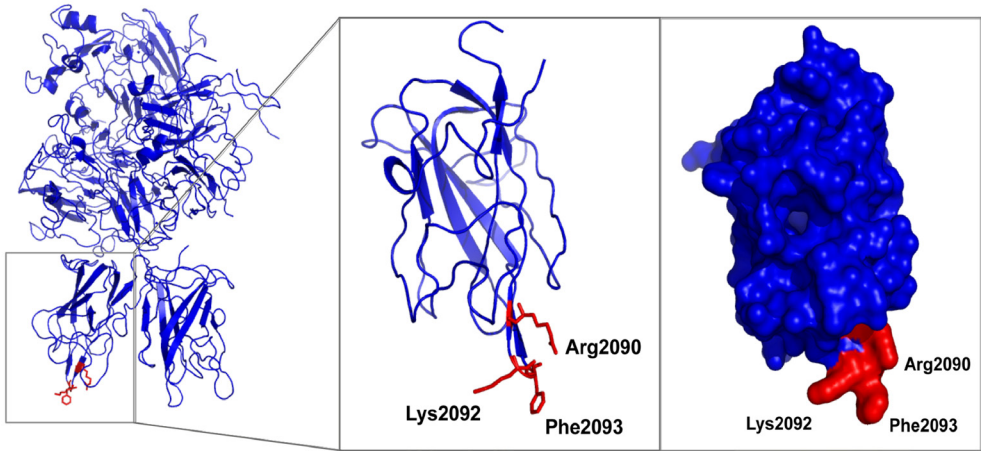

Figure 2. Structural properties of the C1 domain of FVIII.
A surface representation of the C1 domain of FVIII was prepared with Pymol imaging program (DeLano Scientific, Palo Alto, CA). Residues 2090,2092 and 2093 are indicated in red.

FVIII with alanine substitutions in positions 2090, 2092 and 2093 shows diminished binding to KM33

It was previously shown that C1 domain residues 2092 and 2093 are required for KM33 binding to FVIII[19]. However, since mutating these residues to alanines does not completely abolish KM33 binding, we hypothesize that additional C1 domain amino acids can be involved in the interaction between FVIII and KM33. To test this hypothesis, we mutated an additional positively charged amino acid present at the tip of the C1 domain (Figure 2). Arginine at position 2090 was changed to alanine. To establish whether such a mutation will have an additional effect, a FVIII variant was prepared where all three residues, 2090, 2092 and 2093, were substituted with alanines. Mutants and wild-type FVII were expressed in HEK293 cells and purified by immunoaffinity chromatography. To explore the binding of purified variants to KM33, we employed a solution-phase sandwich ELISA in which FVIII, bound to immobilized KM33, was detected either via an A2 or C2 domain targeting antibody (CLB-CAg9 or CLB-CAg117, respectively). As expected, FVIII 2092/2093 showed a significant reduction in ability to bind KM33 as compared to wild-type FVIII. Introduction of a single alanine substitution in position 2090 did not influence the binding, however the presence of this mutation additional to mutations in 2092/2093 showed an even more pronounced decrease in binding to KM33 (Figure 3A). To validate these findings, we performed surface plasmon resonance analysis of the FVIII-KM33 interaction. 25 nM full-length KM33 (IgG1) was passed over FVIII WT, FVIII 2092/2093 and FVIII 2090/2092/2093 as described in the Materials and Methods section. Similar to the previous observation, FVIII 2090/2092/2093 showed greater reduction of binding

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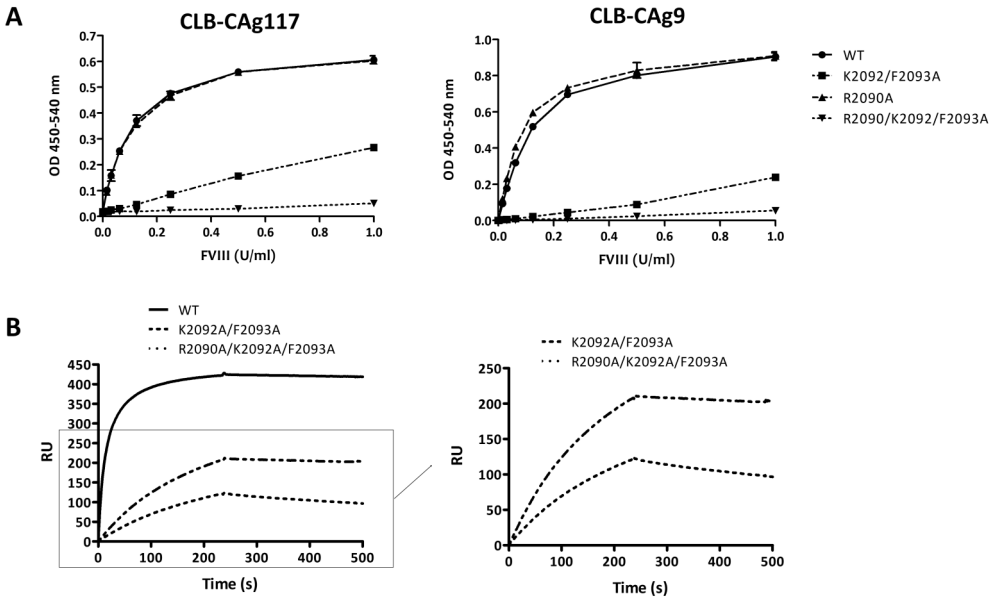

Figure 3. FVIII with alanine substitutions in positions 2090, 2092 and 2093 shows diminished binding to KM33

A,B. KM33 was immobilized on microtiter plates and incubated with increasing concentrations of wild-type (circles) or mutants: 2090 (triangles), 2092/93 (squares) or 2090/92/93 (diamonds). Bound FVIII was detected either with C2 domain-directed antibody CLB-CAg117 **(A)** or A2 domain-directed antibody CLB-CAg9 **(B)**. Data are averages of 2 experiments and presented as % maximum binding (maximum binding corresponds to the optical density measured for the highest concentration of FVIII wild-type). **C.** Surface Plasmon resonance analysis of FVIII binding to KM33. 25 nM KM33 IgG1 was passed over FVIII wild-type (solid line), FVIII 2092/93 (dashed line) or FVIII 2090/92/93 (dotted line) as described in detail in the Materials and Methods section. Data are representative for two experiments.

C1 domain mutations alter FVIII endocytosis by human antigen-presenting cells

As previously shown, KM33 abrogates the uptake of FVIII by human monocyte-derived dendritic cells, and residues 2090, 2092 and 2093 are important for binding of KM33 to FVIII. These findings suggest that this surface-exposed region in the C1 domain may be of great importance for endocytosis of FVIII by antigen-presenting cells (Figure 2). To investigate this, BMDCs were incubated for 30 minutes with 15 nM FVIII wild-type or mutants, then fixed and stained with CLB-CAg117 antibody in presence of saponine, and internalized FVIII was subsequently detected by flow cytometry.

FVIII R2090A was endocytosed at a slightly reduced level as compare to FVIII wild-type (Figure 4), whereas endocytosis of FVIII 2092/2093 was more severely impaired. Uptake of FVIII 2090/2092/2093 was even more reduced when compared to FVIII-K2092A-F2093A (Figure 4). These results show that Arg2090, Lys2092 and Phe2093 contribute to the uptake of FVIII by murine bone marrow derived dendritic cells.

The role of C1 domain in modulation of immune responses to FVIII *in vivo*

So far, our data suggest, that residues 2090, 2092 and 2093 are important for cellular uptake of FVIII by murine antigen-presenting cells. We investigated whether reduced recognition and endocytosis of a FVIII variant that contains alanine substitutions at these three positions will also lead to reduced *in vivo* immune responses in terms of activation of FVIII-specific T cells and B cells as well as subsequent anti-FVIII antibody production.

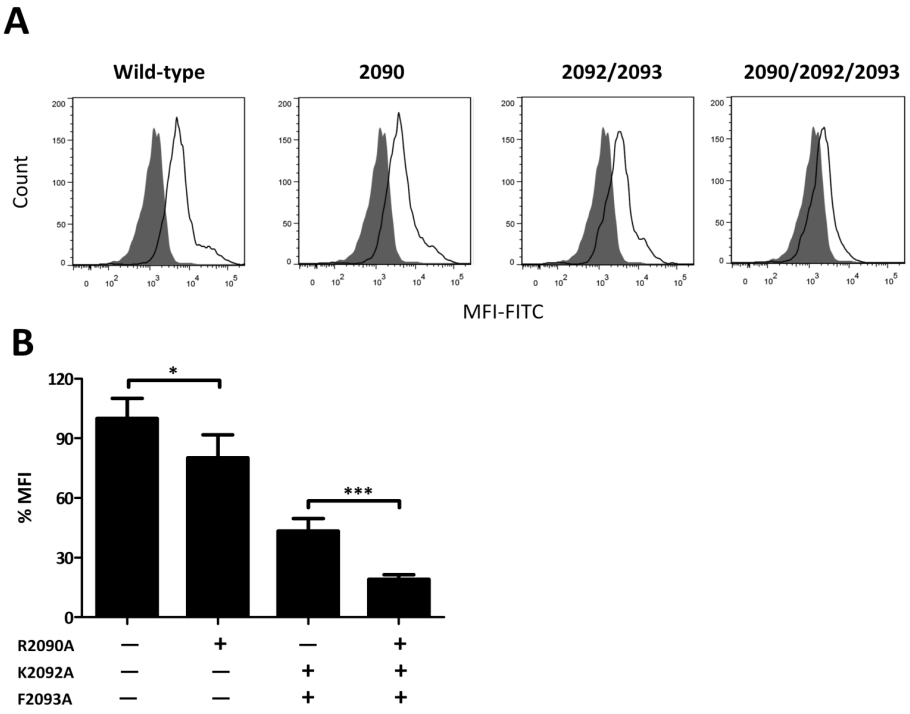

Figure 4. Endocytosis of FVIII by murine bone-marrow derived dendritic cells is mediated by C1 residues 2090, 2092 and 2093.

15 nM of FVIII wild-type or mutants: 2090, 2092/93 or 2090/92/93 was added to BMDCs for 30 minutes at 37°C. Internalization was detected by addition of CLB-CAg117-FITC in the presence of saponin. Endocytosis was measured by flow cytometry. Grey filled histograms represent untreated cells. Data show mean ± SD from at least 3 independent experiments. Mean fluorescence was shown in histograms **(A)** or bar diagram **(B)**.

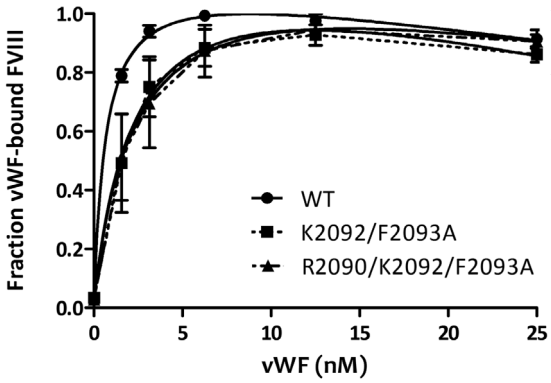

Figure 5. Binding of FVIII variants to vWF.
Anti vWF antibody CLB-Rag20 (2.5 µg/ml) was immobilized on microtiter plates. vWF (0-0.5 U/ml) was first preincubated with 1 U/ml of wild-type (circles) or mutants: 2092/93 (squares) or 2090/92/93 (triangles) at 15 min, 37°C and then added to RAg20 coated plate. FVIII bound to the plate was detected using CLB-CAg117-HRP.

We first established that the FVIII variants were capable of binding to von Willebrand factor (vWF), to exclude the possibility that differences in immunogenicity of FVIII variants were due to differences in vWF binding (Figure 5). *In vitro* experiments showed that FVIII with alanine substitutions for all three tested residues: 2090, 2092 and 2093, showed the most reduction in endocytosis. Therefore, we decided to test this variant *in vivo*. $FVIII^{-/-}$ mice (E17 KO) were injected weekly with 1 µg of either FVIII wild-type or FVIII 2090/92/93. After 5 consecutive injections, the plasmas of mice injected with wild-type FVIII have a high anti-FVIII antibody titer, while the titer was significantly lower in the group that received FVIII 2090/92/93 (Figure 6A and 6B). Additionally, we performed an ELISpot assay, to visualize anti-FVIII antibody-secreting cells (ASCs) present in the spleen of injected mice. In agreement with the antibody titers in the plasma, mice injected with wild-type FVIII had significant number of anti-FVIII ASCs, producing mainly IgG1 or IgG2b antibodies, whereas the group that received 2090/92/93 variant had little or no anti-FVIII antibody-secreting cells (Figure 6C). Moreover, injection of mice with 2090/92/93 FVIII variant led to significantly reduced proliferation of splenic $CD4^+$ T cells upon *in vitro* restimulation with FVIII (Figure 6D and 6E). Together, these results suggests that specific modification of FVIII leading to reduction in its endocytosis by antigen-presenting cells, is an effective way to reduce FVIII immunogenicity *in vivo*.

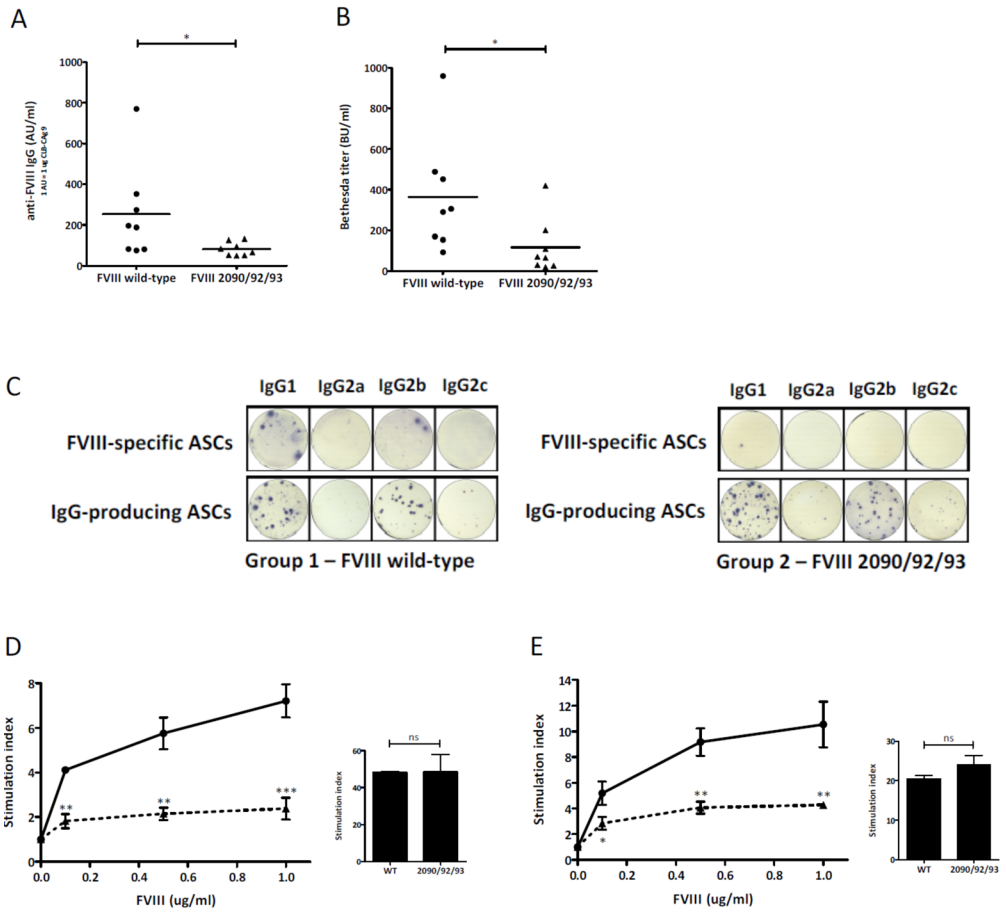

Figure 6. Reduced immune responses upon *in vivo* administration of FVIII 2090/92/93 in FVIII$^{-/-}$ mice.

A,B. Hemophilic E17 KO mice (n=8) were injected intravenously 5 times weekly with 1 µg of FVIII wild-type or 2090/92/93. One week after the last injections, mice were sacrificed and blood samples were collected. Anti-FVIII antibody titers from plasma samples were measured by ELISA **(A)** and Bethesda assay **(B)** as described in detail in the Materials and Methods section. *P<0.05 (nonparametric Mann-Whitney U test). **C.** Single cell suspensions of spleens collected from injected mice were pooled within groups. The presence of antibody-secreting cells (ASCs) producing anti-FVIII antibodies was determined by ELISpot. As a control, total IgG-producing ASCs were detected. Representative wells, displaying both ASC types are shown for both groups. **D,E.** Pooled splenocytes from each group were depleted of CD8^{+} cells by magnetic bead separation and remaining CD8^{-} cells were assayed in a thymidine (^{3}H)incorporation assay after stimulation with increasing concentrations of FVIII wild-type (circles) or mutant 2090/2092/2093 (triangles). Proliferation was measured after 72 **(D)** or 96 hours **(E)** and thymidine was added for the last 18-20 hours. Results are shown as stimulation index (counts with antigen/counts without antigen) from triplicate wells (mean ±SD) for both FVIII-specific (left panel) as well as nonspecific (right panel) proliferation. ns - not significant, *P<0.05, **P<0.01, ***P<0.001 (Student's t-test).

Discussion

In this study we demonstrate that the endocytosis of FVIII by murine antigen-presenting cells is mediated by C1-domain residues Arg 2090, Lys 2092 and Phe 2093. Substitution of these three residues to alanine residues resulted in a FVIII variant that retains its procoagulant activity, but has an almost complete reduction in endocytosis by human as well as murine dendritic cells. After injection into hemophilic mice, it induces significantly lower inhibitor titers as compared to wild-type FVIII.

We have previously shown that monoclonal antibody KM33 blocks the endocytosis of FVIII by human monocyte-derived DCs[18]. In this study, we confirm that KM33 blocks FVIII endocytosis by murine DCs. FVIII residues Lys 2092 and Phe 2093 are the two residues mainly involved in the interaction with KM33.[25] Binding of KM33 to this region also inhibits LRP-mediated endocytosis of FVIII by U87MG cells.[25] Here, we confirm that FVIII with alanine substitutions at positions 2092 and 2093 has reduced binding to KM33 and additionally show that alanine substitution of Arg at position 2090 decreases the binding to KM33 even further. Consequently, we show that endocytosis of such FVIII variants is also affected for murine BMDCs, with the most pronounced effect of FVIII 2090/92/93. Finally, repeated injections of modified FVIII variant leads to significant decrease in anti-FVIII antibody titers as compared to the wild-type FVIII, which correlates with reduced frequency of both FVIII-specific $CD4^+$ splenic T cells and FVIII-specific antibody-secreting plasma cells.

The immunogenicity of FVIII has previously been shown to be inversely associated with its binding to vWF.[33] Binding of FVIII to vWF protects FVIII from endocytosis by human DCs. However, in agreement with previous reports[34], 2090/92/93 variant is capable of binding to vWF only with a slightly reduced affinity as compared to wild-type FVIII (Figure 5). Nevertheless, this would not explain its reduction in immunogenicity, as reduction in vWF binding should lead to enhanced endocytosis of FVIII by DCs. In another recent publication, the immunogenicity of FVIII was shown to be indirectly linked to its procoagulant activity.[35] Thrombin, the end-product of the coagulation cascade, is capable of providing APCs with the "danger" signal which is a necessary stimulus for APCs to activate $CD4^+$ T cells. Mice treated with a specific inhibitor of thrombin, hirudin, developed lower inhibitor titers after subsequent treatment with FVIII. It is important to establish whether the reduction of immunogenicity displayed by the 2090/92/93 variant in this study is due to a reduction in endocytosis rather than a reduction in its procoagulant activity and therefore a reduced ability to provide APCs with a "danger" signal. However, 2090/92/93 variant displays similar activity levels to wild-type FVIII as measured by a chromogenic Factor Xa generation assay, with specific activity ranging from 6300 to 8800 U/mg.

Since endocytosis of FVIII is diminished by introducing C1 domain substitutions, the 2090/92/93 variant could also be a useful tool for further identification of receptor(s) used by APCs to internalize FVIII. Macrophage mannose receptor was proposed as an important interaction partner

of FVIII.[13] FVIII contains multiple potential glycosylation sites with two major residues described to expose mannose-ending glycosylations located on the heavy (N239) and the light chain (N2118).[36] However, none of the FVIII variant tested in this study has modified glycans, yet their endocytosis is significantly impaired. This argues against mannose receptor being a major receptor for FVIII endocytosis by antigen-presenting cells. Broadly expressed scavenger receptor LRP and other LDL receptor family members are reported as important receptors for FVIII clearance[37,38], however their role in FVIII endocytosis by DCs has been refuted.[18,39] Residues 2092 and 2093 have been shown to play a role in FVIII binding to LRP.[25] Disruption of FVIII-LRP binding could potentially lead to prolonged half-life time of FVIII *in vivo*.

In summary, we show that a FVIII variant with three alanine substitutions in the C1 domain, at positions 2090, 2092 and 2093, is strikingly less immunogenic than normal FVIII both *in vitro* and *in vitro*. The reduction in immunogenicity is a consequence of a reduced ability of different APC cell types to recognize and internalize this FVIII variant. This variant retains significant procoagulant activity, which makes this variant therapeutically interesting for the treatment of hemophilia A patients.

References

1. Leissinger CA. Prophylaxis in haemophilia patients with inhibitors. *Haemophilia*. 2006;12 Suppl 6:67-73.
2. Brackmann HH, Gormsen J. Massive factor-VIII infusion in haemophiliac with factor-VIII inhibitor, high responder. *Lancet*. 1977;2(8044):933.
3. Lollar P. Pathogenic antibodies to coagulation factors. Part one: factor VIII and factor IX. *J Thromb Haemost*. 2004;2(7):1082-1095.
4. Dimichele D. The North American Immune Tolerance Registry: contributions to the thirty-year experience with immune tolerance therapy. *Haemophilia*. 2009;15(1):320-328.
5. Bohn RL, Avorn J, Glynn RJ, Choodnovskiy I, Haschemeyer R, Aledort LM. Prophylactic use of factor VIII: an economic evaluation. *Thromb Haemost*. 1998;79(5):932-937.
6. Qian J, Collins M, Sharpe AH, Hoyer LW. Prevention and treatment of factor VIII inhibitors in murine hemophilia A. *Blood*. 2000;95(4):1324-1329.
7. Reipert BM, Sasgary M, Ahmad RU, Auer W, Turecek PL, Schwarz HP. Blockade of CD40/CD40 ligand interactions prevents induction of factor VIII inhibitors in hemophilic mice but does not induce lasting immune tolerance. *Thromb Haemost*. 2001;86(6):1345-1352.
8. Wu H, Reding M, Qian J, et al. Mechanism of the immune response to human factor VIII in murine hemophilia A. *ThrombHaemost*. 2001;85(1):125-133.
9. Ragni MV, Bontempo FA, Lewis JH. Disappearance of inhibitor to factor VIII in HIV-infected hemophiliacs with progression to AIDS or severe ARC. *Transfusion*. 1989;29(5):447-449.
10. Jones TD, Phillips WJ, Smith BJ, et al. Identification and removal of a promiscuous CD4+ T cell epitope from the C1 domain of factor VIII. *J Thromb Haemost*. 2005;3(5):991-1000.
11. James EA, Kwok WW, Ettinger RA, Thompson AR, Pratt KP. T-cell responses over time in a mild hemophilia A inhibitor subject: epitope identification and transient immunogenicity of the corresponding self-peptide. *J Thromb Haemost*. 2007;5(12):2399-2407.
12. James EA, van Haren SD, Ettinger RA, et al. T-cell responses in two unrelated hemophilia A inhibitor subjects include an epitope at the factor VIII R593C missense site. *J Thromb Haemost*. 2011;9(4):689-699.
13. Dasgupta S, Navarrete AM, Bayry J, et al. A role for exposed mannosylations in presentation of human therapeutic self-proteins to CD4+ T lymphocytes. *Proc Natl Acad Sci U S A*. 2007;104(21):8965-8970.
14. Bi L, Lawler AM, Antonarakis SE, High KA, Gearhart JD, Kazazian HH, Jr. Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A. *Nat Genet*. 1995;10(1):119-121.
15. van Schooten CJ, Shahbazi S, Groot E, et al. Macrophages contribute to the cellular uptake of von Willebrand factor and factor VIII in vivo. *Blood*. 2008;112(5):1704-1712.
16. Navarrete A, Dasgupta S, Delignat S, et al. Splenic marginal zone antigen presenting cells are critical for the primary allo-immune response to therapeutic factor VIII in hemophilia A. *J Thromb Haemost*. 2009;7(11):1816-1823.
17. Matsui H, Shibata M, Brown B, et al. A murine model for induction of long-term immunologic tolerance to factor VIII does not require persistent detectable levels of plasma factor VIII and involves contributions from Foxp3+ T regulatory cells. *Blood*. 2009;114(3):677-685.
18. Herczenik E, van Haren SD, Wroblewska A, et al. Uptake of blood coagulation factor VIII by dendritic cells is mediated via its C1 domain. *J Allergy Clin Immunol*. 2011. In Press.

19. Meems H, Meijer AB, Cullinan DB, Mertens K, Gilbert GE. Factor VIII C1 domain residues Lys 2092 and Phe 2093 contribute to membrane binding and cofactor activity. *Blood*. 2009;114(18):3938-3946.
20. Brinkman HJ, Mertens K, van Mourik JA. Phospholipid-binding domain of factor VIII is involved in endothelial cell-mediated activation of factor X by factor IXa. *Arterioscler Thromb Vasc Biol*. 2002;22(3):511-516.
21. Boel E, Verlaan S, Poppelier MJ, Westerdaal NA, Van Strijp JA, Logtenberg T. Functional human monoclonal antibodies of all isotypes constructed from phage display library-derived single-chain Fv antibody fragments. *JImmunolMethods*. 2000;239(1-2):153-166.
22. Saeland E, Vidarsson G, Leusen JH, et al. Central role of complement in passive protection by human IgG1 and IgG2 anti-pneumococcal antibodies in mice. *J Immunol*. 2003;170(12):6158-6164.
23. Mertens K, Donath MJ, van Leen RW, et al. Biological activity of recombinant factor VIII variants lacking the central B-domain and the heavy-chain sequence Lys713-Arg740: discordant in vitro and in vivo activity. *Br J Haematol*. 1993;85(1):133-142.
24. Fribourg C, Meijer AB, Mertens K. The interface between the EGF2 domain and the protease domain in blood coagulation factor IX contributes to factor VIII binding and factor X activation. *Biochemistry*. 2006;45(35):10777-10785.
25. Meems H, van den Biggelaar M, Rondaij M, van der Zwaan C, Mertens K, Meijer AB. C1 domain residues Lys 2092 and Phe 2093 are of major importance for the endocytic uptake of coagulation factor VIII. *Int J Biochem Cell Biol*. 2011;43(8):1114-1121.
26. Donath MS, Lenting PJ, Van Mourik JA, Mertens K. The role of cleavage of the light chain at positions Arg1689 or Arg1721 in subunit interaction and activation of human blood coagulation factor VIII. *J BiolChem*. 1995;270(8):3648-3655.
27. Bi L, Sarkar R, Naas T, et al. Further characterization of factor VIII-deficient mice created by gene targeting: RNA and protein studies. *Blood*. 1996;88(9):3446-3450.
28. Muchitsch EM, Turecek PL, Zimmermann K, et al. Phenotypic expression of murine hemophilia. *ThrombHaemost*. 1999;82(4):1371-1373.
29. Reipert BM, Ahmad RU, Turecek PL, Schwarz HP. Characterization of antibodies induced by human factor VIII in a murine knockout model of hemophilia A. *ThrombHaemost*. 2000;84(5):826-832.
30. Lutz MB, Kukutsch N, Ogilvie AL, et al. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *JImmunolMethods*. 1999;223(1):77-92.
31. Verbruggen B, Novakova I, Wessels H, Boezeman J, van den BM, Mauser-Bunschoten E. The Nijmegen modification of the Bethesda assay for factor VIII:C inhibitors: improved specificity and reliability. *ThrombHaemost*. 1995;73(2):247-251.
32. Slifka MK, Antia R, Whitmire JK, Ahmed R. Humoral immunity due to long-lived plasma cells. *Immunity*. 1998;8(3):363-372.
33. Dasgupta S, Repesse Y, Bayry J, et al. VWF protects FVIII from endocytosis by dendritic cells and subsequent presentation to immune effectors. *Blood*. 2007;109(2):610-612.
34. Lu J, Pipe SW, Miao H, Jacquemin M, Gilbert GE. A membrane-interactive surface on the factor VIII C1 domain cooperates with the C2 domain for cofactor function. *Blood*. 2010.
35. Skupsky J, Zhang AH, Su Y, Scott DW. A role for thrombin in the initiation of the immune response to therapeutic Factor VIII. *Blood*. 2009;114(21):4741-4748.

36. Medzihradszky KF, Besman MJ, Burlingame AL. Structural characterization of site-specific N-glycosylation of recombinant human factor VIII by reversed-phase high-performance liquid chromatography-electrospray ionization mass spectrometry. *Anal Chem*. 1997;69(19):3986-3994.
37. Schwarz HP, Lenting PJ, Binder B, et al. Involvement of low-density lipoprotein receptor-related protein (LRP) in the clearance of factor VIII in von Willebrand factor-deficient mice. *Blood*. 2000;95(5):1703-1708.
38. Cunningham N, Laffan MA, Manning RA, O'Donnell JS. Low-density lipoprotein receptor-related protein polymorphisms in patients with elevated factor VIII coagulant activity and venous thrombosis. *Blood Coagul Fibrinolysis*. 2005;16(7):465-468.
39. Dasgupta S, Navarrete AM, Andre S, et al. Factor VIII bypasses CD91/LRP for endocytosis by dendritic cells leading to T-cell activation. *Haematologica*. 2008;93(1):83-89.

Chapter 5

HLA-DR-presented peptide-repertoires derived from human monocyte-derived dendritic cells pulsed with blood coagulation factor VIII

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Abstract

Activation of T-helper cells is dependent upon the appropriate presentation of antigen-derived peptides on MHC class II molecules expressed on antigen-presenting cells. In the current study we explored the repertoire of peptides presented on MHC class II molecules on human monocyte-derived dendritic cells (moDCs) from a panel of four HLA-typed healthy donors. MHC class II-bound peptides could be routinely recovered from small cultures containing $5x10^6$ cells. A fraction of the identified peptides were derived from proteins localized in the plasma membrane, endosomes and lysosomes, but the majority of peptides that were presented on MHC class II originate from other organelles. Subsequently, we studied the antigen-specific peptide repertoire after endocytosis of a soluble antigen. Blood coagulation factor VIII (FVIII) was chosen as the model antigen since our current knowledge on MHC class II presented peptides derived from this immunogenic therapeutic protein is limited. Analysis of the total repertoire of MHC class II-associated peptides revealed that per individual sample 20-50 FVIII-derived peptides were presented on FVIII-pulsed moDCs. Repertoires of FVIII-derived peptides eluted from moDCs derived from four HLA typed donors revealed that some MHC class II-presented FVIII peptides were presented by multiple donors, while the presentation of other FVIII peptides was donor-specific. In total 32 different core peptides were presented on FVIII-pulsed moDCs from 4 HLA-typed donors. Together our findings provide an unbiased approach to identify peptides that are presented by MHC class II on antigen-loaded moDCs from individual donors.

Introduction

Antigen-presenting cells (APCs) continuously process endogenous and exogenous antigens into small peptides that are loaded on MHC class I or MHC class II molecules for presentation to T lymphocytes.[1] Classically, endogenous antigens are presented on MHC class I molecules for presentation to $CD8^+$ T cells whereas peptides derived from exogenous, internalized antigens are loaded on MHC class II molecules activate $CD4^+$ T cells. Over the last decade this concept has been successfully challenged. Firm proof has been obtained for the presentation of exogenous antigens on MHC class I molecules for cross-priming of $CD8^+$ T cells.[2] Similarly, inspection of the repertoire of naturally occurring peptides presented on MHC class II revealed that the majority of the presented peptides is in fact derived from endogenous proteins.[3,4] Not surprisingly, a large proportion of naturally presented peptides are derived from proteins that reside in endosomes or lysosomes.[3,4] Recent studies suggest that resident proteins of non-endocytic compartments, such as mitochondria or the nucleus, can also be presented on MHC class II by sampling of intracellular compartments through autophagy.[5-8] Current efforts to probe the repertoire of antigen-derived naturally presented peptides are limited by the number of cells needed to obtain substantial amounts of MHC class II bound peptide. Until now the repertoire of naturally presented peptides has been mainly explored using panels of well-characterized immortalized B cells. Typically, around $5x10^9$ cells are used for sample preparation.[9-11] More recently, MHC class II-presented peptides have been successfully isolated from tissue specimens of patients with multiple sclerosis.[12] An elegant study by Wahlstrom and co-workers used human bronchial lavage cells from a pool of patients with sarcoidosis to obtain information on antigenic peptides involved in the pathogenesis of this disease.[13] Further advances in MHC peptide identification and quantification by mass spectrometry have already led to the identification of large numbers of MHC class I peptides from more limiting amounts of cells[14] and has allowed for functional analysis regarding the role of the immunoproteasome in the generation of MHC class I peptides.[15]

The aim of this study was to investigate whether a significant amount of MHC class II-presented peptides can be eluted from small cultures of human moDCs. MoDCs are professional APCs that express high levels of MHC class II that become surface-exposed following their maturation.[16] Our results indicate that several hundred MHC class II-bound peptides can be eluted from samples containing as few as $5x10^6$ moDCs. This allows for the analysis of MHC class II presented peptides from 50 milliliter blood drawn from individual donors. We subsequently investigated whether pulsing moDCs with an antigen resulted in the presentation of antigen derived peptides on MHC class II. Blood coagulation factor VIII (FVIII) was used as a model antigen for this study. Therapeutic administration of FVIII is used to correct the bleeding tendency of hemophilia A patients who lack functional FVIII.[17] Up to 25% of patients with hemophilia A develop high affinity antibodies in response to infusion of FVIII, which are also referred to as "inhibitors".[18] FVIII inhibitors are mostly high-affinity IgG antibodies, which are the result of FVIII-specific

T-cell activation by professional APCs, followed by T-cell dependent antibody class-switching and affinity maturation.[19] At present our knowledge on the repertoire of naturally presented FVIII derived peptides is limited. Here we show that from each individual donor between 20 and 50 partially overlapping FVIII-specific peptides could be recovered from moDCs, corresponding to 8-17 different potential $CD4^+$ T-cell epitopes. These findings demonstrate that small numbers of moDCs can be used to probe the repertoire of presented peptides of potentially immunogenic antigens such as FVIII.

Materials and Methods

Subjects

Blood was drawn from HLA-typed healthy volunteers in accordance with Dutch regulations and following approval from Sanquin Ethical Advisory Board in accordance with the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were isolated from freshly drawn, EDTA anticoagulated blood by separation over a Ficoll-Paque PLUS gradient (d = 1.077, GE Healthcare,Uppsala, Sweden).

Reagents

In this study, the following reagents were used: Recombinant human FVIII (Advate) was kindly provided by Dr. B.M. Reipert (Baxter Healthcare Corporation, Vienna, Austria), CD14 microbeads (MACS, Miltenyi Biotech Bergisch Gladbach, Germany), anti-CD80-FITC, anti-CD83-APC, anti-CD86-APC (BD Biosciences, USA) and anti-CD14-PE (Sanquin Reagents, Amsterdam, the Netherlands). Cellgro DC serum-free medium, IL-4 and GM-CSF were obtained from CellGenix (Freiburg, Germany). LPS was obtained from Sigma-Aldrich (St. Louis, USA). Hybridoma L243 (anti-HLA-DR) was obtained from ATCC (Wesel, Germany).

MoDC preparation and factor VIII endocytosis

Monocytes were isolated from the PBMC fraction by positive selection using CD14 microbeads and a magnetic cell separator (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the monocytes isolated was determined by flow cytometry analysis.[20] Monocytes were cultured at a concentration of 0.83×10^6 cells/ml in a 6 well plate (Nunc, Roskilde Denmark) in Cellgro medium supplemented with GM-CSF (1000 IU/ml) and IL-4 (800 IU/ml) for 5 days.[20] After 5 days of culture, the immature moDCs were washed and replated in Cellgro medium supplemented with GM-CSF and IL-4 at a concentration of 2.5×10^6 cells/ml in a final volume of 2 ml. Cells were incubated with 100 nM FVIII for 5h prior to induction of maturation. After 5h, the immature moDCs were maturated using 1 μg/ml LPS for 24 h in the presence of 1% human serum. The adherent maturated moDCs were detached by 5 minute incubation with phosphate buffered saline (PBS) containing 0.25% trisodiumcitrate and washed before analysis.

Flow cytometric analysis of cell-surface phenotype

For determining the phenotype of moDCs, immature moDCs or mature moDCs were washed with PBS containing 0.5% bovine serum albumin (PBS/0.5%BSA) and incubated with 50 μl 1 μg/ml mAb or appropriate isotype controls diluted in PBS/0.5%BSA for 30 min at 4°C. Cells were washed twice and resuspended in PBS/0.5%BSA. 4',6-diamidino-2-phenylindole (DAPI) was added to the cells before analysis to assess cell viability and exclude dead cells from analysis. Cells were analyzed on an LSRII flow cytometer (Beckton Dickinson, San Jose, USA) and analyzed with Flowjo software version 7.5.5 (Tree Star, Inc, Ashland, USA).

Purification of HLA-DR presented peptides on moDCs

HLA-DR molecules were purified from FVIII-treated, maturated moDCs or PBS treated control moDCs essentially as described previously.[9] Briefly, moDC pellets were resuspended in 50 mM Tris pH 7.0 containing 4% Igepal CA-630 (Sigma, St. Louis, USA). The membrane fraction was solubilised by end-over-end incubation at 4°C for 30 minutes. HLA-DR was purified from the detergent-soluble fraction by immuno-affinity chromatography using antibody L243-coupled to CNBr Sepharose 4B (Amersham Biosciences, Buckinghamshire, UK) in the presence of protease inhibitors (Complete Protease Inhibitor Cocktail Tablet, 1 tablet per 50 ml buffer, Roche Diagnostics GmbH, Mannheim, Germany) overnight at 4°C. After washing the Sepharose 5 times with 50 mM Tris-HCl pH 7.0, peptides were eluted from HLA-DR by incubation with 10% acetic acid for 15 minutes at 70°C. Eluted peptides were purified from the acetic acid eluate using a C18 ziptip (Millipore, Billerica, USA). In parallel experiments, cell lysates were incubated with isotype control antibody-coupled Sepharose (clone CLB-T4/1, mouse IgG2a, Sanquin Reagents, Amsterdam, The Netherlands).

Analysis of peptides by mass spectrometry

Eluted peptides were separated using a reversed-phase C18 column (50 µm x 20 cm, 5 µm particles) (Nanoseparations, Nieuwkoop, The Netherlands), at a flowrate of 100 nl/min with a one hour gradient from 0% to 35% (v/v) acetonitrile with 0.1% HAc. Eluted peptides were sprayed directly into the LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific Inc, Bremen, Germany) using a nanoelectrospray source with a spray voltage of 1.9 kV. The LTQ was operated in a data-dependent mode by performing collision induced dissociation in the ion-trap (35% normalized collision energy) for the five most intensive precursor ions selected from each full scan in the Orbitrap (300-2000 m/z, resolving power 30.000). An isolation width of 2 Da was used for the selected ions (charge ≥2) and an activation time of 30 ms. Dynamic exclusion was activated for the MS/MS scan with a repeat count of 1 and exclusion duration of 30 s. To obtain a high mass accuracy, the LTQ Orbitrap was calibrated on a monthly basis using a calibration solution consisting of caffeine, MRFA and Ultramark 1621 as recommended by the manufacturer.

Peptide identification

Peptides were identified using a Sequest search algorithm against UniprotKB non-redundant protein database 25.H_sapiens.fasta (53,784 non-redundant entries actually searched), utilizing Proteome Discoverer release version 1.1 software (Thermo Scientific, Bremen, Germany).[21] Identification of peptides was performed using the following filter settings. During the Sequest search, we allowed a mass deviation of 20 ppm. In general mass deviations were below 3 ppm. Fragment mass tolerance was 0.8 Da. All peptides with a charge state of 2 have a minimal XCorr score of 2.0. For peptides with charge state 3, the minimal XCorr score is 2.25. For charge state 4, 2.5; charge state 5, 2.75; charge state 6, 3.0; charge state 7, 3.2 and charge state >7, 3.4. All peptides not complying to these criteria were excluded. Annotated spectra of individual

peptides described in this manuscript are provided in the online supplementary information which can be found here: http://www.mcponline.org/content/10/6/M110.002246/suppl/DC1

Validation of MHC class II bound peptides by differential expression analysis

MoDCs were prepared from donor A as described above. Duplicate endocytosis experiments were conducted on $2*10^6$ cells, instead of $5*10^6$ cells. Cells were incubated with either 100 nM FVIII or with PBS as a control. Immune precipitations were performed as described above, using either L234-coupled Sepharose (anti-MHC class II) or Sepharose coupled with an isotype control antibody. Each sample was analyzed separately by mass spectrometry. SIEVE™ release version 1.2.0 software (Thermo Scientific, Bremen, Germany) was used to compare the duplicate experiments and subsequently analyze differences in peptide abundance between the different conditions. All frames were analyzed with retention time between 20 and 50 minutes and m/z between 300 and 2000. Peak intensity threshold was set at 100000. For peptide identification, the same sequest criteria were used as described above.

Results

Quantitative analysis of MHC class II-bound peptides.

In order to study the peptide repertoire presented on MHC class II, we used moDCs from HLA-typed healthy donors. MoDCs were analysed for the presence of CD14, CD80, CD83, CD86 and MHC class II on the surface (Figure 1A). Immature moDCs were defined as CD14 negative and were also checked for the absence of maturation markers CD80, CD83 and CD86. MHC class II was present on immature moDCs. Cells that were incubated with LPS were positive for maturation markers and showed an increased surface expression of MHC class II (Figure 1B). FVIII is endocytosed by moDCs, leading to activation of FVIII-specific $CD4^+$ T cells.[22] We confirm that FVIII is efficiently taken up by immature moDCs (Figure 1C).

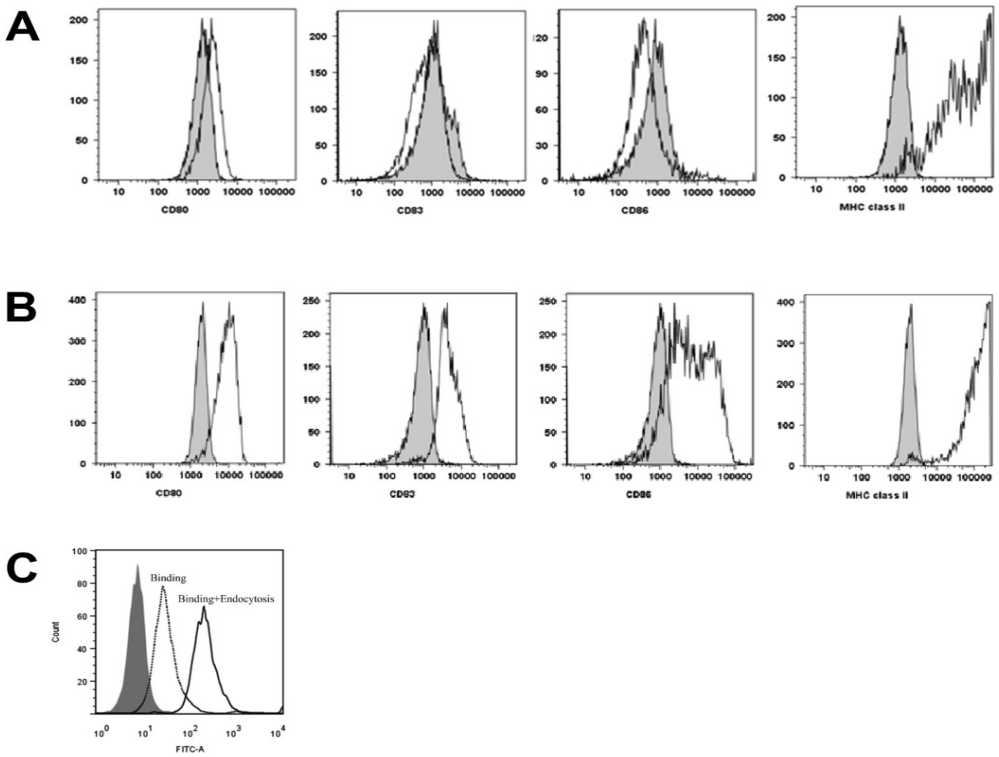


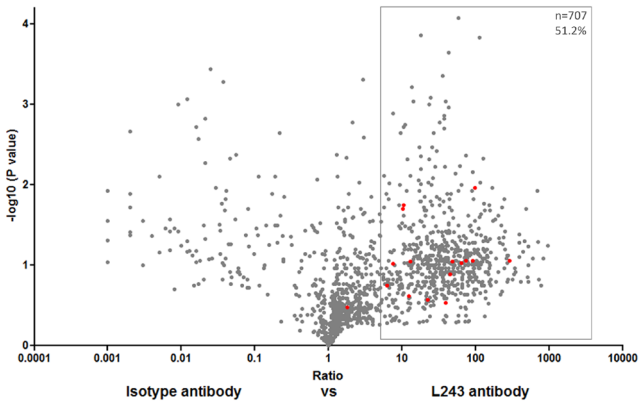
Figure 1. Cell surface markers of immature and mature moDCs.

Cells were analyzed at the immature **(A)** and mature **(B)** state for the presence of cell surface markers CD80, CD83, CD86 and MHC class II. Grey histograms indicate isotype controls. A representative culture is depicted. **(C)** Endocytosis and presentation of FVIII by moDCs. Histogram shows the fluorescence intensity for a FITC-labeled anti FVIII antibody (CLB-CAg-117). Binding of CLB-CAg-117-FITC to untreated cells is shown in grey. Cells stained in the absence of saponin show the membrane binding of FVIII (dotted curve). Increase of fluorescence in cells stained in the presence of saponin indicates that FVIII was successfully internalized. A representative graph of one of the four donors is depicted.

For the analysis of MHC class II-bound peptides, HLA-DR molecules were purified from cell lysates of mature moDCs using anti-HLA-DR antibody L243 coupled to Sepharose. HLA-DR-bound peptides were subsequently obtained as described in the Experimental procedures. The sequence of the eluted HLA-DR ligands was determined by LC-MS.

In order to verify to which extent our method generates specific MHC class II-bound peptides, immunoprecipitations were performed on FVIII-treated moDC samples from donor A (DRB1*0101 and DRB1*1301), using L243-Sepharose, which were compared with control immunoprecipitations, using an isotype control antibody coupled to Sepharose. The relative abundance of peptides between these experiments was compared using SIEVE 1.2. differential analysis software.

A



B

(FVIII residues) Sequence	Ratio
(0242-0259) LPGLIGCHRKSVYWHVIG	22.532
(0457-0474) VGDTLLIIFKNQASRPYN	10.258
(1454-1472) GDQREVGSLGTSATNSVTY	97.518
(1526-1540) LQGTEGAIKWNEANR	73.612
(1690-1720) SFQKKTRHYFIAAVERLWDYGMSSSPHVLRN	1.786
(1723-1739) KTRHYFIAAVERLWD	7.482
(1767-1783) VEDNIMVTFRNQASRPY	12.514
(1769-1783) DNIMVTFRNQASRPY	10.466
(1994-2008) GIWRVECLIGEHLHA	39.100
(1995-2008) IWRVECLIGEHLHA	6.320
(2098-2111) ISQFIIMYSLDGKK	12.845
(2098-2112) ISQFIIMYSLDGKKW	44.828
(2192-2207) ASSYFTNMFATWSPSK	63.806
(2309-2320) HPQSWVHQIALRM	286.885
(2309-2322) HPQSWVHQIALRMEV	91.546
(2309-2323) HPQSWVHQIALRMEVL	48.385

Figure 2. Identification and relative quantification of MHC class II-bound peptides. **(A)** Volcano plot representation showing detected peptide ions across duplicate analyses. MoDCs from donor A (DRB1*0101, 1301) were used. FVIII-treated cells were immunoprecipitated using either an anti-MHC class II antibody or an isotype control antibody. Experiments were performed in duplicates. SIEVE was used to compare intensities of individual peptides eluted from L243 or isotype-control Sepharose. Peptide clusters highlighted in the box on the right hand side were considered as MHC class II-bound peptides (fold change ≥5). Only peptides that were sequenced by MS/MS are depicted. FVIII-derived peptides are indicated in red. The sequence and ratio of all FVIII peptides are shown in panel **(B)**.

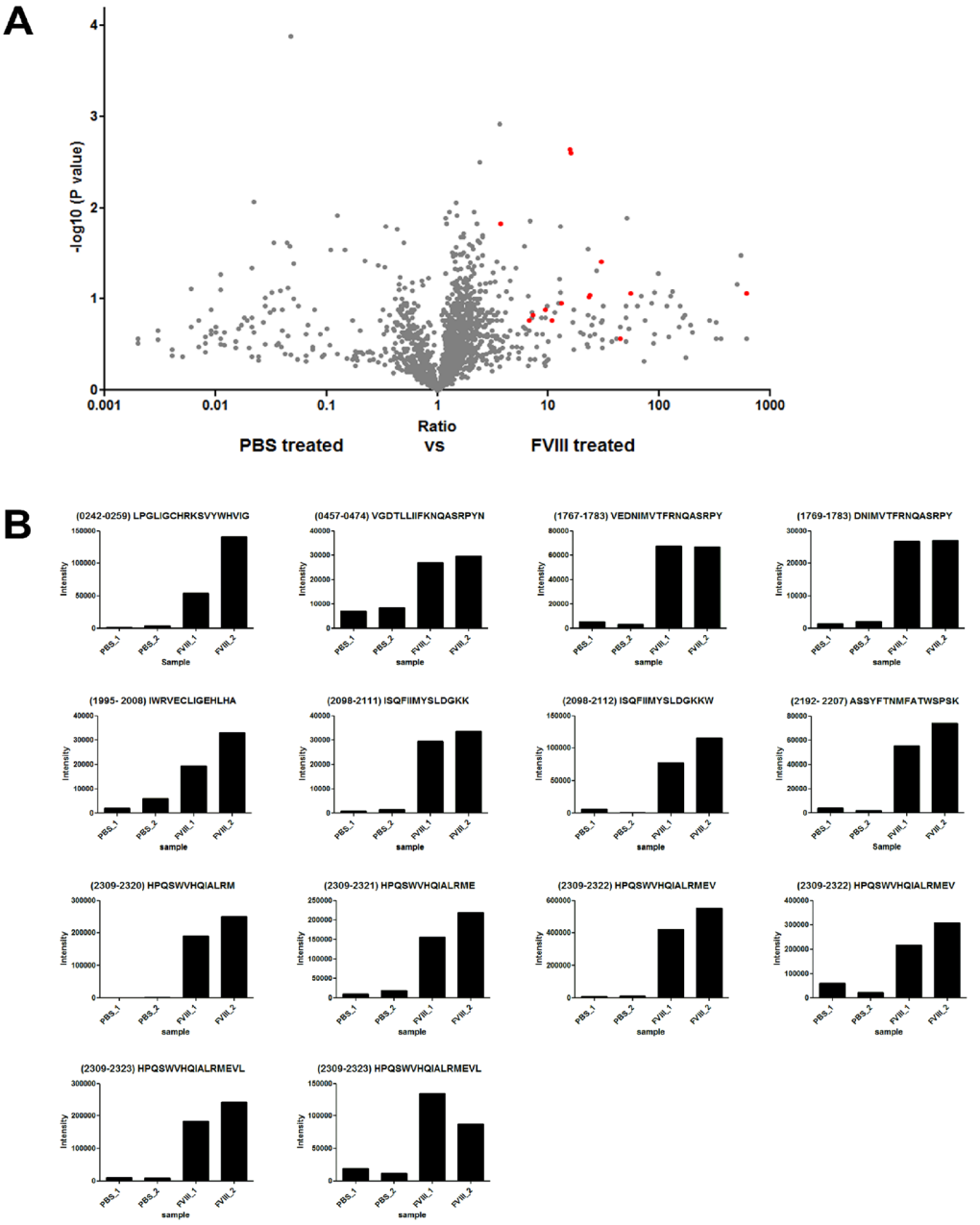


Figure 3. Identification and relative quantification of MHC class II-bound FVIII peptides.
(continued on next page)

Figure 3. Identification and relative quantification of MHC class II-bound FVIII peptides.
(A) Volcano plot representation showing detected peptide ions across duplicate analyses. MoDCs from donor A (DRB1*0101, 1301) were used. Cells were treated with either 100 nM FVIII or with PBS. All samples were immunoprecipitated using an anti-MHC class II antibody. Experiments were performed in duplicates. SIEVE was used to compare intensities of individual peptides obtained from FVIII and PBS treated cells. Only peptides that were derived from MS2 spectra are depicted. Peptides identified as FVIII-derived peptides are indicated in red. **(B)** Peak intensities of all identified FVIII peptides are shown for experimental and control samples. It should be noted that the sets of FVIII peptides that were identified in the analysis presented in Figure 3B when compared to Figure 2B are not completely identical. The reason for the observed differences between Figure 2B and 3B is that these particular peptides were filtered out by the algorithms employed by SIEVE during the comparison, due to local spectral differences. Manual analysis of the raw data files revealed that all peptides depicted in Figure 2B and 3B were indeed present in the duplicate samples prepared from FVIII-treated cells using L243 (MHC class II specific) Sepharose.

Figure 2A clearly shows that the majority of peptides have a ratio L243:isotype higher than 1, meaning that these peptides are more abundantly present when samples are immunoprecipitated with L243-Sepharose as compared to the samples where isotype-Sepharose was used. Peptides were regarded as specific MHC class II ligands when the ratio was above 5. Using this stringent threshold, many peptides are regarded as non-specific. All FVIII peptides, however, with the exception of one peptide, are specific MHC class II ligands (Figure 2A and 2B). Absolute identification of peptides present in samples immunoprecipitated with the control-Sepharose does, however, identify additional FVIII peptides. These peptides are all derived from the same region in FVIII. This set of non-specific Sepharose-binding peptides is reproducible for all donors tested (data not shown). The fact that even these peptides may be enriched in samples immunoprecipitated with L243 could mean that, additional to their background binding to control-Sepharose, they also contain an MHC class II-binding motif. This is also shown in Figure 2A, where a FVIII peptide, containing the same sequence as the one identified as non-specific, has a ratio of 7.482. Peptides with this sequence were excluded from all subsequent analyses. To further illustrate that peptides in Figure 2A with a ratio larger than 5 are in fact true MHC class II-associated peptides, the Propred prediction algorithm was used to compare the predicted binding properties of all peptides with a ratio larger than 5 with peptides that have a ratio less than 5.[23] Online Supplementary Figure 1 shows that peptides with a ratio larger than 5 are predicted to bind significantly better to both MHC class II alleles of donor A (DRB1*0101 and DRB1*1301) than peptides with a ratio below 5.
Differential expression analysis of PBS-treated moDCs versus FVIII-treated moDCs, using SIEVE 1.2 software, revealed that all FVIII peptides were identified as specific for the FVIII-treated cells as seen in Figure 3A and B. This validates the correct identification of FVIII peptides. Peak intensities from individual FVIII peptides obtained for duplicate samples of PBS- and FVIII-treated moDCs revealed that FVIII peptides are markedly less present in samples derived from PBS-treated moDCs (Figure 3B).

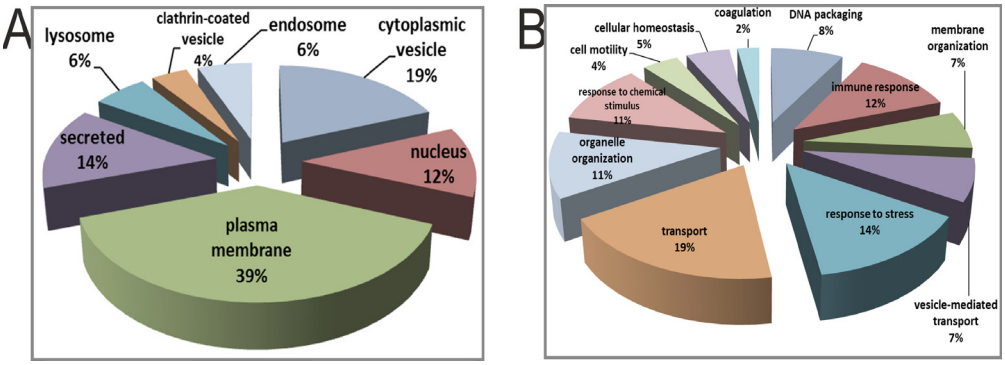


Figure 4. Subcellular and functional categorization of source proteins.
All proteins of which peptides with a ratio ≥5 were identified in Figure 2A (specific MHC class II-bound peptides) were annotated based on subcellular localization or function using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) Bioinformatics Resource. (**A**) pie chart shows the percentages of proteins found presented on MHC class II according to their subcellular localization. **(B)** pie chart shows the functional association of these proteins.

Analysis of MHC class II-presented peptides.
Figure 2A clearly shows that most peptides that are presented on MHC class II are not derived from FVIII. We therefore analyzed which other proteins are presented as well. All peptides in Figure 2A with a ratio larger than 5 were subsequently identified using Sequest and annotated based on subcellular localization (Figure 4A) and function (Figure 4B), using the online functional annotation tool DAVID v6.7 (http://david.abcc.ncifcrf.gov).[24,25] Many peptides presented on HLA-DR are derived from proteins that reside in compartments which are part of the MHC class II-presentation pathway, such as plasma membrane, cytosol and lysosomes. However, proteins from other cellular compartments, such as the nucleus (12%), and secreted vesicles (14%). Many of the endogenously presented peptides are derived from proteins that are functionally involved in intracellular transport (19%), immune response (12%) and stress response (14%) (Figure 4B).

Analysis of MHC class II-presented FVIII peptides.
In the previous paragraph we have demonstrated that limited numbers of moDCs suffice to detect a significant part of the repertoire of peptides presented on HLA-DR after endocytosis of FVIII. We used moDCs derived from four different donors to further study the repertoire of naturally presented FVIII peptides. All FVIII-derived peptides identified comply to the XCorr scores described in the Experimental procedures. In addition, FVIII peptides were excluded when they were identified as aspecific binders according to the method described in Figure 2A. FVIII peptides could be divided into sets of peptides with overlapping sequences which were

FVIII	peptide	Charge	Xcorr	RT (min)	m/z (Da)	?M (ppm)	Propred (DRB1*0101/*1301) (max=1)	NetMHCIIpan (DRB1*0101/*1301) (affinity nM)
0107-0136 (A1)	KASEGAEYDDQTSQREKEDDKVFPGGSHTY	5	3.47	29.14	675,706	-1.80	- / 0.011 VFPGGSHTY	3124.05 VFPGGSHTY / 17365.72 VFPGGSHTY
0242-0256 (A1)	LPGLIGCHRKSVYWH	4	1.53	33.77	442,238	-2.03	- / 0.3860 LIGCHRKSVY	371.18 LIGCHRKSV / 14589.46 LIGCHRKSV
0242-0258 (A1)	LPGLIGCHRKSVYWHVI	3	2.70	37.85	660,032	-2.19		
0242-0259 (A1)	LPGLIGCHRKSVYWHVIG	4	1.87	37.46	509,531	-2.43		
0245-0258 (A1)	LIGCHRKSVYWHVI	3	1.56	33.94	570,979	-2.25		
0288-0301 (A1)	ISPITFLTAQTLLM	2	2.62	56.65	774,939	-0.24	0.2333 ITFLTAQTL / 0.2614 FLTAQTLLM	13.35 FLTAQTLLM / 2430.27 ITFLTAQTL
0288-0304 (A1)	ISPITFLTAQTLLMDLG	2	2.81	56.71	917,505	-0.56		
0457-0474 (A2)	VGDTLLIIFKNQASRPYN	3	3.41	44.93	683,709	-0.12	0.080 LIIFKNQAS / 0.3977 LIIFKNQAS	22.38 FKNQASRPY / 178.47 LIIFKNQAS
0459-0473 (A2)	DTLLIIFKNQASRPY	3	1.68	45.04	593,666	2.27		
1098-1112 (B)	KMLFLPESARWIQRT	3	1.80	40.23	626,012	-2.25	0.2817 FLPESARWI / 0.1136 FLPESARWI	13.04 FLPESARWI / 14548.48 LFLPESARW
1122-1140 (B)	GPSPKQLVSLGPEKSVEG	3	2.54	34.33	603,662	-2.18	0.1833 LVSLGPEKS / 0.3750 LVSLGPEKS	16.34 LVSLGPEKS / 9797.54 LVSLGPEKS
1205-1222 (B)	IQENVVLPQIHTVTGTKN	4	1.90	33.12	498,520	-16.26	- / 0.3295 VVLPQIHTV	89.21 IHTVTGTKN / 4626.90 LPQIHTVTG
1489-1503 (B)	SGKVELLPKVHIYQK	4	2.21	32.37	435,510	-1.88	0.0667 VELLPKVHI / 0.3750 VELLPKVHI	101.93 VELLPKVHI / 769.65 VELLPKVHI
1594-1608 (B)	KKDTILSLNACESNH	3	3.08	32.25	558,281	-1.89	0.1333 TILSLNACES / 0.3068 TILSLNACES	57.98 ILSLNACES / 16751.17 TILSLNACE
1594-1609 (B)	KKDTILSLNACESNHA	4	2.39	32.17	436,722	-2.14		
1594-1610 (B)	KKDTILSLNACESNHAI	4	2.02	35.18	464,993	-2.45		
1767-1783 (A3)	VEDNIMVTFRNQASRPY	3	2.82	39.97	680,669	-1.78	0.2317 FRNQASRPY / 0.4205 FRNQASRPY	26.07 MVTFRNQAS / 1893.23 MVTFRNQAS
1767-1784 (A3)	VEDNIMVTFRNQASRPYS	3	2.49	39.42	709,680	-1.96		
1768-1783 (A3)	EDNIMVTFRNQASRPY	3	2.39	39.74	647,646	-2.28		
1769-1783 (A3)	DNIMVTFRNQASRPY	3	2.46	39.52	604,633	-0.25		
1801-1818 (A3)	EPRKNFVKPNETKTYFWK	5	1.56	31.17	463,248	-2.55	- / 0.2955 VKPNETKTY	43.45 FVKPNETKT / 5833.05 VKPNETKTY
1989-2009 (A3)	LPSKAGIWRVECLIGEHLHAG	4	2.53	43.68	572,308	-3.10	- / 0.1250 LIGEHLHAG	
1993-2008 (A3)	AGIWRVECLIGEHLHA	3	3.73	44.01	601,982	0.09		
1994-2008 (A3)	GIWRVECLIGEHLHA	4	1.33	43.59	433,979	-0.76		
1994-2009 (A3)	GIWRVECLIGEHLHAG	4	1.80	43.11	448,234	-1.68		
1995-2008 (A3)	IWRVECLIGEHLHA	4	3.13	42.51	419,723	-1.96		
1995-2009 (A3)	IWRVECLIGEHLHAG	4	2.91	41.99	433,978	-1.81		
2097-2112 (C1)	YISQFIIMYSLDGKKW	3	2.04	48.89	664,683	1.56	0.3833 FIIMYSLDG / 0.3523 FIIMYSLDG	5.76 FIIMYSLDG / 2329.60 FIIMYSLDG
2098-2111 (C1)	ISQFIIMYSLDGKK	3	3.32	42.92	548,300	-1.74		
2098-2112 (C1)	ISQFIIMYSLDGKKW	2	2.83	46.24	914,985	-1.65		
2145-2159 (C1)	IARYIRLHPTHYSIR	5	1.86	29.12	380,020	-1.58	0.2817 YIRLHPTHY / 0.7841 IRLHPTHYS	17.48 YIRLHPTHY / 1959.92 YIRLHPTHY
2190-2207 (C2)	ITASSYFTNMFATWSPSK	2	3.75	51.72	1.019,987	4.48	0.1817 FTNMFATWS / 0.0568 FTNMFATWS	13.17 FTNMFATWS / 16184.61 ASSYFTNMF
2192-2207 (C2)	ASSYFTNMFATWSPSK	2	2.67	48.38	912,915	-2.14		
2204-2222 (C2)	SPSKARLHLQGRSNAWRPQ	5	2.86	26.38	438,640	-3.21	- / 0.2841 LHLQGRSNA	275.89 LHLQGRSNA / 3447.67 LHLQGRSNA
2204-2223 (C2)	SPSKARLHLQGRSNAWRPQV	5	1.62	28.69	458,455	-2.19		
2308-2321 (C2)	IHPQSWVHQIALRM	4	2.47	39.80	429,734	-2.01	0.4633 WVHQIALRM / 0.3864 WVHQIALRM	5.22 WVHQIALRM / 480.09 WVHQIALRM
2309-2321 (C2)	HPQSWVHQIALRM	3	3.26	37.60	534,948	-2.43		
2309-2322 (C2)	HPQSWVHQIALRME	3	3.23	37.76	577,962	-2.11		
2309-2323 (C2)	HPQSWVHQIALRMEV	3	3.36	40.42	610,985	-2.16		
2309-2324 (C2)	HPQSWVHQIALRMEVL	3	3.27	43.67	648,679	-2.39		
2309-2325 (C2)	HPQSWVHQIALRMEVLG	3	3.24	43.45	667,688	0.49		

Figure 5. FVIII-derived MHC class II ligands identified from donor A.

The first column shows residue numbers and corresponding domain of the FVIII molecule. The following columns show amino acid sequence, charge, XCorr value as provided by Sequest, retention time (RT), mass-to-charge ratio (m/z) and mass tolerance (ΔM). The two last columns display predicted binding scores to the MHC class II alleles of the donor. Binding scores to corresponding MHC class II alleles of the donor were calculated using Propred and NetMHCIIpan epitope prediction programs. Thresholds were defined as ≤ 500 nM for NetMHCIIpan and as top 3% natural binders for Propred (≥ 0.023 for DRB1*0101 and ≥ 0.295 for DRB1*1301). The motif of peptides that are predicted to bind above the threshold are underlined.

FVIII	Experiment 1	Experiment 2	Propred (DRB1*0701/*1501) (max=1)	NetMHCIIpan (DRB1*0701/*1501) (affinity nM)
0001-0036 (A1)		ATRRYYLGAVELSWDYMQSDLGELPVDARFPPRVPK	0.4397 YMQSDLGEL / 0.0918 LPVDARFPP	794.87 YMQSDLGEL / 215.13 RYYLGAVEL
0001-0036 (A1)		ATRRYYLGAVELSWDYMQSDLGELPVDARFPPRVPK		
0107-0136 (A1)		KASEGAEYDDQTSQREKEDDKVFPGGSHTY	0.1379 VFPGGSHTY / 0.1429 VFPGGSHTY	15433.83 VFPGGSHTY / 12015.44 VFPGGSHTY
0361-0380 (a1-A2)	DDDNSPSFIQIRSVAKKHPK		0.2845 FIQJRSVAK / 0.0918 IQJRSVAKK	41.87 FIQJRSVAK / 24.21 FIQJRSVAK
0362-0380 (a1-A2)	DDNSPSFIQIRSVAKKHPK			
0364-0379 (a1-A2)	NSPSFIQIRSVAKKHP	NSPSFIQIRSVAKKHP		
0364-0380 (a1-A2)	NSPSFIQIRSVAKKHPK			
0365-0380 (a1-A2)	SPSFIQIRSVAKKHPK	SPSFIQIRSVAKKHPK		
0455-0474 (A2)	GEVGDTLLIIFKNQASRPYN		0.2759 VGDTLLIIFK / 0.5204 LIIFKNQAS	22.26 FKNQASRPY / 4.09 LIIFKNQAS
0455-0475 (A2)		GEVGDTLLIIFKNQASRPYNI		
0456-0473 (A2)		EVGDTLLIIFKNQASRPY		
0456-0474 (A2)	EVGDTLLIIFKNQASRPYN	EVGDTLLIIFKNQASRPYN		
0456-0475 (A2)	EVGDTLLIIFKNQASRPYNI	EVGDTLLIIFKNQASRPYNI		
0456-0479 (A2)		EVGDTLLIIFKNQASRPYNIYPHG		
0457-0473 (A2)	VGDTLLIIFKNQASRPY	VGDTLLIIFKNQASRPY		
0457-0474 (A2)	VGDTLLIIFKNQASRPYN	VGDTLLIIFKNQASRPYN		
0457-0475 (A2)	VGDTLLIIFKNQASRPYNI	VGDTLLIIFKNQASRPYNI		
0458-0473 (A2)	GDTLLIIFKNQASRPY	GDTLLIIFKNQASRPY	0.25 LLIIFKNQA / 0.5204 LIIFKNQAS	
0459-0472 (A2)		DTLLIIFKNQASRP		358.16 LIIFKNQAS / 4.09 LIIFKNQAS
0459-0473 (A2)	DTLLIIFKNQASRPY	DTLLIIFKNQASRPY		
0459-0474 (A2)	DTLLIIFKNQASRPYN	DTLLIIFKNQASRPYN		
0459-0475 (A2)		DTLLIIFKNQASRPYNI		
0467-0489 (A2)	NQASRPYNIYPHGITDVRPLYSR	NQASRPYNIYPHGITDVRPLYSR	0.2328 IYPHGITDV / 0.3163 YNIYPHGIT	1097.78 NQASRPYNI / 2800.65 ITDVRPLYS
0724-0740 (a2)	EDISAYLLSKNNAIEPR	EDISAYLLSKNNAIEPR	0.6638 YLLSKNNAI / 0.2857 YLLSKNNAI	3.69 YLLSKNNAI / 88.41 YLLSKNNAI
0725-0739 (a2)	DISAYLLSKNNAIEP	DISAYLLSKNNAIEP		
0725-0740 (a2)	DISAYLLSKNNAIEPR	DISAYLLSKNNAIEPR		
0726-0739 (a2)	ISAYLLSKNNAIEPR	ISAYLLSKNNAIEPR		
0986-0999 (B)	KDNALFKVSISLLK	KDNALFKVSISLLK	0.4138 FKVSISLLK / 0.1888 LFKVSISLL	10.02 FKVSISLLK / 324.82 FKVSISLLK
1227-1240 (B)	LFLLSTRQNVEGSY		0.4741 LFLLSTRQNV /0.1735 LFLLSTRQNV	191.78 FLLSTRQNV / 659.61 LFLLSTRQN
1223-1235 (B)		FMKNLFLLSTRQN		
1765-1783 (A3)	AEVEDNIMVTFRNQASRPY		0.3103 FRNQASRPY / 0.4694 MVTFRNQAS	100.48 FRNQASRPY / 15.68 MVTFRNQAS
1765-1784 (A3)	AEVEDNIMVTFRNQASRPYS			
1766-1783 (A3)	EVEDNIMVTFRNQASRPY			
1766-1784 (A3)	EVEDNIMVTFRNQASRPYS			
1767-1782 (A3)	VEDNIMVTFRNQASRP		0.1983 IMVTFRNQA / 0.4694 MVTFRNQAS	2236.73 MVTFRNQAS / 15.68 MVTFRNQAS
1767-1783 (A3)	VEDNIMVTFRNQASRPY	VEDNIMVTFRNQASRPY		
1767-1784 (A3)	VEDNIMVTFRNQASRPYS	VEDNIMVTFRNQASRPYS		
1768-1782 (A3)	EDNIMVTFRNQASRP			
1768-1783 (A3)	EDNIMVTFRNQASRPY	EDNIMVTFRNQASRPY		
1768-1784 (A3)	EDNIMVTFRNQASRPYS			
1769-1783 (A3)	DNIMVTFRNQASRPY	DNIMVTFRNQASRPY		
1769-1784 (A3)	DNIMVTFRNQASRPYS			
1816-1827 (A3)		FWKVQHHMAPTK	0.0776 FWKVQHHMA / 0.1122 VQHHMAPTK	2591.59 VQHHMAPTK / 206.74 VQHHMAPTK
1879-1895 (A3)		FFTIFDETKSWYFTEN	0.2586 FDETKSWYF / 0.1837 FTIFDETKS	29.76 FDETKSWYF / 8108.35 FDETKSWYF
1880-1894 (B)	FTIFDETKSWYFTE	FTIFDETKSWYFTE		
1881-1894 (B)	TIFDETKSWYFTE	TIFDETKSWYFTE	0.2586 FDETKSWYF / -	
1881-1895 (B)		TIFDETKSWYFTEN		
1882-1895 (B)	IFDETKSWYFTEN			
2050-2061 (C1)	LARLHYSGSINA		0.4397 LHYSGSINA/ 0.3469 LHYSGSINA	170.98 LHYSGSINA / 18.73 LHYSGSINA
2256-2271 (C2)	YVKEFLISSSQDGHQW		0.4397 FLISSSQDG / 0.2398 VKEFLISSS	83.34 FLISSSQDG / 1011.01 FLISSSQDG
2257-2271 (C2)	VKEFLISSSQDGHQW			

Figure 6. Presentation of FVIII peptides in duplicate experiments from donor B.
Table of FVIII-derived MHC class II ligands identified from donor B. The first column shows residue numbers and corresponding domain of the FVIII molecule. The following columns display amino acid sequences of the first and second experiment. The two last columns show predicted binding scores to the MHC class II alleles of the donor. Binding scores to corresponding MHC class II alleles of the donor were calculated using Propred and NetMHCIIpan epitope prediction programs. Thresholds were defined as ≤ 500 nM for NetMHCIIpan and as top 3% natural binders for Propred (≥ 0.353 for DRB1*0701 and ≥ 0.329 for DRB1*1501). The motif of peptides that are predicted to bind above the threshold are underlined.

comprised of differently processed variants of the same core amino acid sequence. The occurrence of these sets is likely due to the trimming of MHC-bound peptides by endo- and exopeptidases whereas the core peptide sequence which binds to the groove is protected from proteolytic degradation.[1] To illustrate this, Figure 5 shows the complete list of FVIII peptides from donor A. Two different MHC prediction algorithms, Propred and NetMHCIIpan, were chosen to compare our results to, with respect to binding to MHC class II alleles DRB1*0101 and DRB1*1301.[23,26] In order to determine the reproducibility of our findings, duplicate experiments were performed using moDCs obtained from donor B from two different blood draws, with a time period of 2 months in between the different blood draws. The duplicate experiments yielded similar sets of FVIII derived peptides (Figure 6). All the core peptide sequences of which three or more variants are found could be identified in both experiments. In total 10 different core sequences were found in the first experiment and 11 in the second experiment. These sequences were also included in peptides that are only present once or twice. No more than two core sequences could be found in the first experiment only and three sequences were unique for the second experiment. All these were core sequences of which only one or two variants were found. All other core sequences were found both in the first and in the second experiment. Overall, between 930 (donor A) and 270 (donor D) MHC class II-associated peptides could be identified; 702 and 882 peptides were identified for donor B and C, respectively. Thirty-two unique core FVIII peptide sequences could be recovered from all donors together. These sequences are distributed through all the domains of the FVIII molecule. The total amount of FVIII core peptide sequences was 17 for donor A, 10 for donor B, 9 for donor C and 8 for donor D (Figure 7). One core sequence was presented on moDCs derived from all four donors. Two core peptides were common in three out of four donors and five core peptides were common between two out of four donors. All other core peptides were donor-specific. The complete list of FVIII-derived peptides from donor A and B, listed in Figure 5 and Figure 6, respectively, also depict the binding score of each peptide for both DRB1 alleles as calculated using the Propred MHC class II prediction server[23] and NetMHCpan.[26]

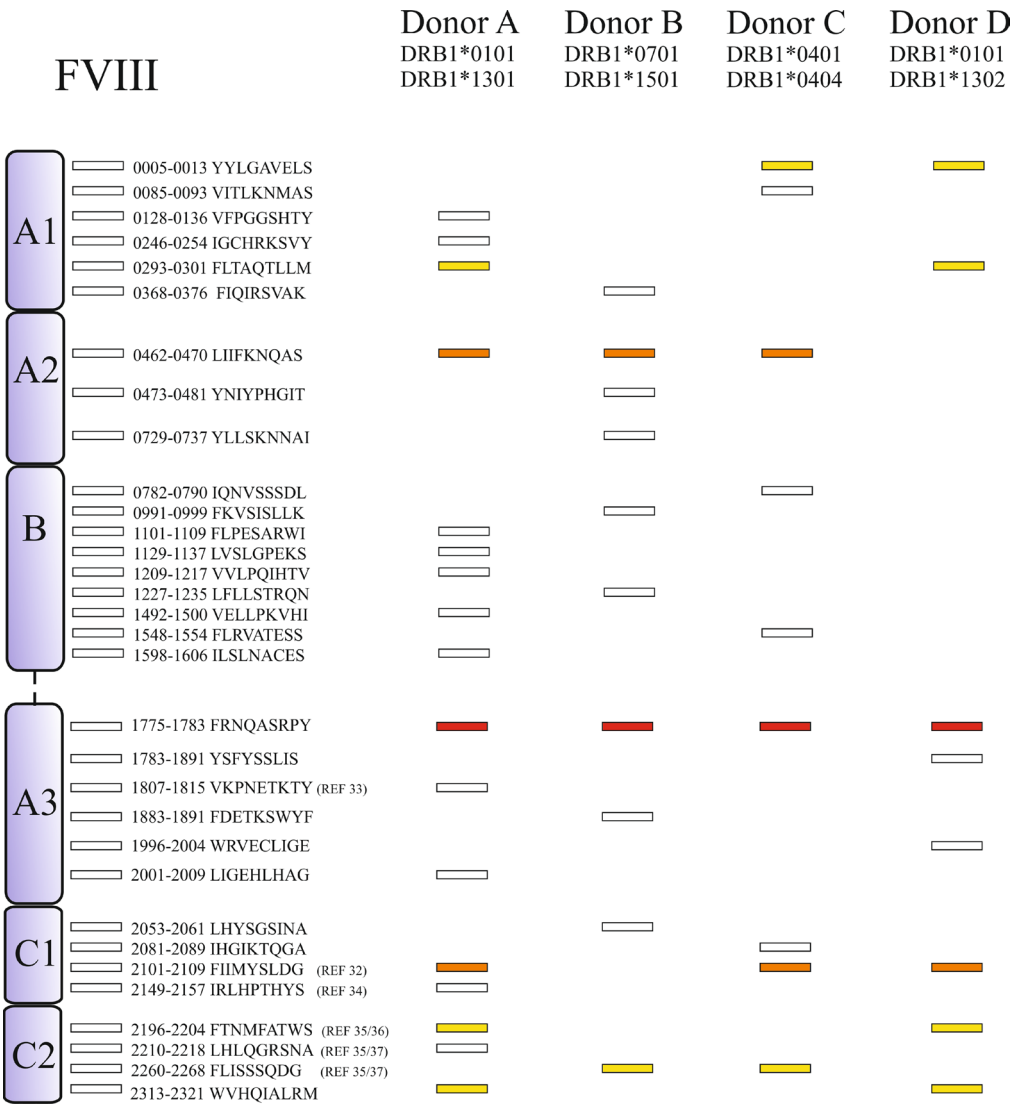


Figure 7. Distribution of FVIII core peptides in 4 different donors.
Different core peptides are presented by different donors. Results displayed in this Figure were obtained from single experiments for all donors. FVIII-derived MHC class II peptides are represented as rectangles for each individual donor. Indicated in yellow are sequences that are common between 2 donors. Shown in orange are sequences common between 3 donors. Displayed in red is a sequence that is common in all four donors. The different domains and the location of the peptides are depicted schematically. Some sequences have been marked with a reference number, indicating that they have been identified previously as $CD4^+$ T-cell epitope in the corresponding publication.

Discussion

In this study we report the analysis of MHC class II-presented peptides on moDCs. Previous reports of MHC class II-presented peptides were performed on cell lines such as B-cell lymfomas, or on tissue-derived material.[5,12] Where most of these studies used 10^9 cells for sample preparation, we performed our experiments with $5x10^6$ moDCs per condition. Our findings show that naturally presented MHC class II peptides can be successfully obtained from from antigen pulsed DC derived of 50 ml of blood. To ensure specificity and reproducibility we performed control experiments, where moDC cell lysates were incubated with Sepharose coupled with an isotype control antibody. Indeed, many peptides were found in the anti-MHC class II immunoprecipitation as well as in the control situation, as seen in Figure 2A. We regarded a peptide a true MHC class II-presented peptide when it was 5 times more abundant in the MHC class II samples as compared to control samples. Using this threshold, almost all FVIII peptides were MHC class II-presented peptides. FVIII peptides that did not fit these criteria were also positively identified in control samples using Sequest peptide identification software. These were excluded from our analysis.

As the data in Figure 2A and 3A clearly indicate, immunoprecipitation of MHC class II-bound peptides from moDC cell reveals that many endogenous proteins are presented on MHC class II. Figure 3A shows that many peptides are presented even when cells are incubated with PBS. Figure 2A shows that 51% of all peptides identified were specific for MHC class II. Most of these peptides are derived from endogenous proteins, and not FVIII. Not only proteins that reside in or are targeted to the endosomal/lysosomal pathway are presented on MHC class II, but proteins from other organelles, such as the nucleus, and cytosol are also presented. Sampling of intracellular compartments, called autophagy, is a constant process responsible for the lysosomal degradation of intracellular proteins, which can subsequently be presented on MHC class II.[8] Professional APCs, such as moDCs used in this study, can enhance the formation of autophagosomes upon triggering of pattern-recognition receptors, such as toll-like receptor 4 (TLR4) and NOD2.[27-29] LPS-induced triggering of TLR4 during maturation of moDCs is likely to promote autophagy under our experimental conditions. Another possible explanation for the high percentage of endogenous proteins presented on MHC class II is the turnover of moDCs during culture. The efficacy of moDC generation from monocytes is about 50%. The other cells undergo apoptosis and can be endocytosed by moDCs. Some of the MHC class II-presented endogenous proteins identified in this study have been shown to be presented on MHC class II previously, such as CD14, α-2-macroglobulin, low-density lipoprotein-related protein 1, macrophage mannose receptor and CD74 (invariant chain)[13] (data not shown). The identification of CD74 in all four donors provides an internal control for the peptide isolation procedure. CD74-derived peptides were expected to be found in each donor as CD74 is associated with newly synthesized MHC class II molecules and occupies the binding groove in the ER to prevent MHC class II loading in the ER.[30] In the endo-lysosomal pathway CD74 is exchanged by other peptides or presented itself on MHC class II.

We observed that moDC derived from four healthy, HLA-typed volunteers all presented FVIII peptides after endocytosis of FVIII. Duplicate experiments from different blood draws (Figure 6) suggest that there is a low level of variability in the presentation of FVIII peptides between independent experiments using cells from the same donor. As shown in Figures 5 and 6, FVIII peptides could be divided into sets of peptides with overlapping sequences which were comprised of differently processed variants of the same core peptide sequence. Almost all FVIII core peptides that were found in this study were predicted to bind to either one or both DRB1 alleles present in the donor in which the core peptides was found. Two MHC class II binding prediction-algorithms, Propred and NetMHCIIpan, were chosen based on the fact that they are reportedly the two most accurate algorithms available[31] and on the fact that other algorithms, such as SYFPEITHI, do not have predictors for all MHC class II haplotypes present in our set of donors.

The comparisons between the peptide identification and the two prediction models does not only confirm that the peptides that were found fit into the binding groove of the corresponding MHC class II molecules, but in addition it also stresses the need for proper identification of potential T-cell epitopes. The prediction values shown for donor A and B in Figure 5 and 6 clearly show that, although all peptides are predicted to bind to at least one of both donor DRB1-molecules, there are many discrepancies between the two different prediction algorithms used. For example, in Figure 5, peptide 1205-1222 QIENVVLPQIHTVTGTKN does not bind to DRB1*0101 and binds quite well to DRB1*1301 according to Propred. According to NetMHCIIpan, however, this peptide binds much better to DRB1*0101 (89 nM) than to DRB1*1301 (4.6 μM). Similar to this peptide, many other peptides can be found in these two Figures where the two algorithms are in disagreement. There are also many examples of peptides where the exact 9 amino acid sequence motif that is supposed to bind into the groove of the DRB1 molecule is different according to one prediction algorithm in comparison to the other. Furthermore, there are many regions in the FVIII sequence that are predicted to bind with high affinity to donor DRB1 molecules, which were not identified in this study. In summary, these observations demonstrate that prediction algorithms alone are not sufficient to accurately predict potential $CD4^+$ T-cell epitopes in FVIII.

Analysis of the FVIII peptide repertoire identified for the four donors revealed that 8 out of 32 core sequences were presented by multiple donors (Figure 7). This suggests that that FVIII contains a number of potential HLA-promiscuous ligands. One such promiscuous ligand, consisting of core sequence FIIMYSLDG, was described previously as an immunodominant epitope using T-cell stimulation assays.[32] Most of the peptides found, however, were donor-specific. It is important to raise the question whether these peptides are clinically relevant. There are a couple of other core sequences, that were identified in this study, which have been reported previously as sequences against which $CD4^+$ T cells were found. A FVIII peptide containing region 1807-1815 was able to induce $CD4^+$ T-cell proliferation in a hemophilia A patient with FVIII inhibitors.[33]

Region 2149-2157 is another core sequence that was reported previously. A CD4+ T-cell clone recognizing this region of FVIII was isolated.[34] Proliferation of this clone has been used by another group as a general readout for FVIII endocytosis by dendritic cells.[22] Region 2196-2204 was identified as a region against which CD4+ T cells are directed both in a study with hemophilic mice[35] and in a study with a hemophilia A patient.[36] This sequence was identified using DRB1*0101 tetramers. Both donors that present this peptide in our study are DRB1*0101 as well. Two other C2 peptides identified in this study were found previously to induce T-cell proliferation.[35,37] The fact that most peptides that were found were donor-specific suggests that these peptides are presented in a HLA-specific context. It is important, however, to keep in mind that the donors are not related and are potentially heterogeneous with respect to the expression of genes involved in antigen presentation. The identification of putative FVIII-derived $CD4^+$ T-cell epitopes is important in order to understand the formation of FVIII-neutralizing antibodies in hemophilia A. As mentioned earlier, there are studies that have reported that $CD4^+$ T cells isolated from hemophilia A patients can proliferate in a FVIII-specific manner.[32-34,38,39] More recently, MHC-class II tetramers have been used to assess the exact epitope, HLA-restriction and the phenotype of FVIII-specific $CD4^+$ T cells.[36,40] These studies have implied a role for $CD4^+$ T cells in inhibitor formation, but there is still very limited data on the repertoire of FVIII peptides that is presented by APCs from donors with different HLA haplotypes. There are various prediction models available that calculate the binding affinity of linear peptides for MHC class II variants using mathematics- or structure-based algorithms[23,41-45], such as the ones used in this study. These prediction models are mathematics- or structure-based methods. They can predict binding motifs with considerable accuracy, but are restricted in the sense that they are based on known peptide-MHC interactions.

An alternative method to identify the binding properties of antigen-derived peptides to MHC variants is to measure the binding of synthetic peptides to recombinant MHC molecules.[46] Combined, these computational and *in vitro* methods accurately define sequences within an antigen that can bind MHC molecules, but these data do not completely reflect the different processes that are involved in antigen presentation. Processes that are important for antigen presentation on MHC class II are route of endocytosis, cleavage by endosomal and lysosomal proteases and antigenic competition. In this study we have taken these processes in consideration by investigating which FVIII peptides are naturally presented on MHC class II in moDCs.

The methods described in this paper can be used to determine the repertoire of naturally presented peptides in antigen-pulsed APC. In this study we show that our approach can be used to obtain information on the repertoire of naturally presented peptides of FVIII. Potentially, this knowledge can be used to design novel less immunogenic FVIII variants that lack promiscuously presented peptides such as FRNQASRPY (A3 domain), FIIMYSLDG (C1 domain) or LIIFKNQAS (A2 domain). We and others show peptide analysis of antigen-pulsed moDCs allows for the identification of naturally presented peptides derived from clinically relevant antigens.[47] Identification of HLA promiscuous sequences within clinically relevant antigens can potentially assist in the design of more efficient vaccines or novel tolerization strategies.

References

1. Trombetta ES, Mellman I. Cell biology of antigen processing in vitro and in vivo. *Annu Rev Immunol*. 2005;23:975-1028.
2. Burgdorf S, Kurts C. Endocytosis mechanisms and the cell biology of antigen presentation. *Curr Opin Immunol*. 2008;20(1):89-95.
3. Chicz RM, Urban RG, Gorga JC, Vignali DA, Lane WS, Strominger JL. Specificity and promiscuity among naturally processed peptides bound to HLA-DR alleles. *J Exp Med*. 1993;178(1):27-47.
4. Lechler R, Aichinger G, Lightstone L. The endogenous pathway of MHC class II antigen presentation. *Immunol Rev*. 1996;151:51-79.
5. Dongre AR, Kovats S, deRoos P, et al. In vivo MHC class II presentation of cytosolic proteins revealed by rapid automated tandem mass spectrometry and functional analyses. *Eur J Immunol*. 2001;31(5):1485-1494.
6. Nedjic J, Aichinger M, Mizushima N, Klein L. Macroautophagy, endogenous MHC II loading and T cell selection: the benefits of breaking the rules. *Curr Opin Immunol*. 2009;21(1):92-97.
7. Dengjel J, Schoor O, Fischer R, et al. Autophagy promotes MHC class II presentation of peptides from intracellular source proteins. *Proc Natl Acad Sci U S A*. 2005;102(22):7922-7927.
8. Schmid D, Pypaert M, Munz C. Antigen-loading compartments for major histocompatibility complex class II molecules continuously receive input from autophagosomes. *Immunity*. 2007;26(1):79-92.
9. Peakman M, Stevens EJ, Lohmann T, et al. Naturally processed and presented epitopes of the islet cell autoantigen IA-2 eluted from HLA-DR4. *J Clin Invest*. 1999;104(10):1449-1457.
10. Seeger FH, Schirle M, Gatfield J, et al. The HLA-A*6601 peptide motif: prediction by pocket structure and verification by peptide analysis. *Immunogenetics*. 1999;49(6):571-576.
11. Krogsgaard M, Wucherpfennig KW, Cannella B, et al. Visualization of myelin basic protein (MBP) T cell epitopes in multiple sclerosis lesions using a monoclonal antibody specific for the human histocompatibility leukocyte antigen (HLA)-DR2-MBP85-99 complex. *J Exp Med*. 2000;191(8):1395-1412.
12. Fissolo N, Haag S, de Graaf KL, et al. Naturally presented peptides on MHC I and II molecules eluted from central nervous system of multiple sclerosis patients. *Mol Cell Proteomics*. 2009;8(9):2090-2101.
13. Wahlstrom J, Dengjel J, Persson B, et al. Identification of HLA-DR-bound peptides presented by human bronchoalveolar lavage cells in sarcoidosis. *J Clin Invest*. 2007;117(11):3576-3582.
14. Fortier MH, Caron E, Hardy MP, et al. The MHC class I peptide repertoire is molded by the transcriptome. *J Exp Med*. 2008;205(3):595-610.
15. de Verteuil D, Muratore-Schroeder TL, Granados DP, et al. Deletion of immunoproteasome subunits imprints on the transcriptome and has a broad impact on peptides presented by major histocompatibility complex I molecules. *Mol Cell Proteomics*. 2010;9(9):2034-2047.
16. van Niel G, Wubbolts R, Ten Broeke T, et al. Dendritic cells regulate exposure of MHC class II at their plasma membrane by oligoubiquitination. *Immunity*. 2006;25(6):885-894.
17. Mannucci PM, Tuddenham EG. The hemophilias--from royal genes to gene therapy. *N Engl J Med*. 2001;344(23):1773-1779.
18. Lollar P. Pathogenic antibodies to coagulation factors. Part one: factor VIII and factor IX. *J Thromb Haemost*. 2004;2(7):1082-1095.
19. Reding MT, Lei S, Lei H, Green D, Gill J, Conti-Fine BM. Distribution of Th1- and Th2-induced anti-factor VIII IgG subclasses in congenital and acquired hemophilia patients. *Thromb Haemost*. 2002;88(4):568-575.

20. Ten Brinke A, Karsten ML, Dieker MC, Zwaginga JJ, van Ham SM. The clinical grade maturation cocktail monophosphoryl lipid A plus IFNgamma generates monocyte-derived dendritic cells with the capacity to migrate and induce Th1 polarization. *Vaccine*. 2007;25(41):7145-7152.
21. Yates JR, 3rd, Eng JK, McCormack AL, Schieltz D. Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein database. *Anal Chem*. 1995;67(8):1426-1436.
22. Dasgupta S, Navarrete AM, Bayry J, et al. A role for exposed mannosylations in presentation of human therapeutic self-proteins to CD4+ T lymphocytes. *Proc Natl Acad Sci U S A*. 2007;104(21):8965-8970.
23. Singh H, Raghava GP. ProPred: prediction of HLA-DR binding sites. *Bioinformatics*. 2001;17(12):1236-1237.
24. Dennis G, Jr., Sherman BT, Hosack DA, et al. DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol*. 2003;4(5):P3.
25. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc*. 2009;4(1):44-57.
26. Nielsen M, Lundegaard C, Blicher T, et al. Quantitative predictions of peptide binding to any HLA-DR molecule of known sequence: NetMHCIIpan. *PLoS Comput Biol*. 2008;4(7):e1000107.
27. Xu Y, Jagannath C, Liu XD, Sharafkhaneh A, Kolodziejska KE, Eissa NT. Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. *Immunity*. 2007;27(1):135-144.
28. Sanjuan MA, Dillon CP, Tait SW, et al. Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. *Nature*. 2007;450(7173):1253-1257.
29. Cooney R, Baker J, Brain O, et al. NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. *Nat Med*. 2010;16(1):90-97.
30. Roche PA, Cresswell P. Invariant chain association with HLA-DR molecules inhibits immunogenic peptide binding. *Nature*. 1990;345(6276):615-618.
31. Lin HH, Zhang GL, Tongchusak S, Reinherz EL, Brusic V. Evaluation of MHC-II peptide binding prediction servers: applications for vaccine research. *BMC Bioinformatics*. 2008;9 Suppl 12:S22.
32. Jones TD, Phillips WJ, Smith BJ, et al. Identification and removal of a promiscuous CD4+ T cell epitope from the C1 domain of factor VIII. *J Thromb Haemost*. 2005;3(5):991-1000.
33. Reding MT, Okita DK, Diethelm-Okita BM, Anderson TA, Conti-Fine BM. Epitope repertoire of human CD4(+) T cells on the A3 domain of coagulation factor VIII. *J Thromb Haemost*. 2004;2(8):1385-1394.
34. Jacquemin M, Vantomme V, Buhot C, et al. CD4+ T-cell clones specific for wild-type factor VIII: a molecular mechanism responsible for a higher incidence of inhibitor formation in mild/moderate hemophilia A. *Blood*. 2003;101(4):1351-1358.
35. Pratt KP, Qian J, Ellaban E, et al. Immunodominant T-cell epitopes in the factor VIII C2 domain are located within an inhibitory antibody binding site. *Thromb Haemost*. 2004;92(3):522-528.
36. James EA, Kwok WW, Ettinger RA, Thompson AR, Pratt KP. T-cell responses over time in a mild hemophilia A inhibitor subject: epitope identification and transient immunogenicity of the corresponding self-peptide. *J Thromb Haemost*. 2007;5(12):2399-2407.
37. Ettinger RA, James EA, Kwok WW, Thompson AR, Pratt KP. HLA-DR-restricted T-cell responses to factor VIII epitopes in a mild haemophilia A family with missense substitution A2201P. *Haemophilia*. 2010;16(102):44-55.
38. Reding MT, Okita DK, Diethelm-Okita BM, Anderson TA, Conti-Fine BM. Human CD4+ T-cell epitope repertoire on the C2 domain of coagulation factor VIII. *J Thromb Haemost*. 2003;1(8):1777-1784.

39. Reding MT, Wu H, Krampf M, et al. CD4+ T cell response to factor VIII in hemophilia A, acquired hemophilia, and healthy subjects. *Thromb Haemost*. 1999;82(2):509-515.
40. Ettinger RA, James EA, Kwok WW, Thompson AR, Pratt KP. Lineages of human T-cell clones, including TH17/TH1 cells, isolated at different stages of anti-factor VIII immune responses. *Blood*. *Blood*. 2009;114(7):1423-1428.
41. Sturniolo T, Bono E, Ding J, et al. Generation of tissue-specific and promiscuous HLA ligand databases using DNA microarrays and virtual HLA class II matrices. *Nat Biotechnol*. 1999;17(6):555-561.
42. Bui HH, Sidney J, Peters B, et al. Automated generation and evaluation of specific MHC binding predictive tools: ARB matrix applications. *Immunogenetics*. 2005;57(5):304-314.
43. Nielsen M, Lundegaard C, Lund O. Prediction of MHC class II binding affinity using SMM-align, a novel stabilization matrix alignment method. *BMC Bioinformatics*. 2007;8:238.
44. Nielsen M, Lund O. NN-align. An artificial neural network-based alignment algorithm for MHC class II peptide binding prediction. *BMC Bioinformatics*. 2009;10:296.
45. Wang P, Sidney J, Dow C, Mothe B, Sette A, Peters B. A systematic assessment of MHC class II peptide binding predictions and evaluation of a consensus approach. *PLoS Comput Biol*. 2008;4(4):e1000048.
46. James EA, Moustakas AK, Bui J, Nouv R, Papadopoulos GK, Kwok WW. The binding of antigenic peptides to HLA-DR is influenced by interactions between pocket 6 and pocket 9. *J Immunol*. 2009;183(5):3249-3258.
47. Mutschlechner S, Egger M, Briza P, et al. Naturally processed T cell-activating peptides of the major birch pollen allergen. *J Allergy Clin Immunol*. 2010;125(3):711-718, 718 e711-718 e712.

Chapter 6

Modulation of FVIII peptide presentation on MHC class II induced by differences in dendritic cell maturation

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Abstract

The formation of inhibitory antibodies directed against coagulation factor VIII (FVIII) is a severe complication in the treatment of hemophilia A patients. The induction of anti-FVIII antibodies is a $CD4^+$ T-cell dependent process. Activation of FVIII-specific $CD4^+$ T cells is dependent on the presentation of FVIII-derived peptides on MHC class II by antigen-presenting cells. Previously, we have shown that peptides from different FVIII domains can be presented. Some HLA-promiscuous peptides regions were shown to be commonly presented between different donors, whereas other regions were presented in a more HLA-restricted manner. The presentation of FVIII peptides depends on whether FVIII is internalized and processed in an appropriate fashion for loading of MHC class II molecules. Several different factors are able to alter the presentation of FVIII peptides. In this study, we show that macrophages present less FVIII peptides on MHC class II as compared to dendritic cells. Complex formation of FVIII with monoclonal antibodies prior to endocytosis also induces changes in the preference for presentation of certain FVIII peptides. The presence of IFN-γ or IL-6 during endocytosis and maturation induces only marginal differences in the presentation of FVIII. However, addition of IL-10 or maturation with a cocktail that consists of IL1β, TNF-α and PGE_2 induces marked differences in the spectrum of FVIII peptides presented. In summary, our findings indicate how the presentation of FVIII is altered under specific circumstances. This information may contribute to the understanding of inhibitor development and to the identification of FVIII-specific T cells in hemophilia A patients.

Introduction

Antigen-presenting cells (APCs), such as dendritic cells (DCs) and macrophages, are phagocytic cells capable of internalizing bacteria, fungi, parasites as well as soluble and particulate antigens. Soluble antigens are captured in endosomes, which provides the first step in antigen processing. An important feature of these cells is that they are capable of balancing the proteolytic activity inside endosome and thereby also the generation of antigenic peptides. Soluble antigens can be targeted to distinct endosomal subsets.[1] APCs are capable of temporarily slowing down the acidification of endosomes, thereby allowing for exchange of material between different endosomal compartments.[2,3] Endocytosis-dependent sorting of proteins into different endosomal subsets largely determines whether the protein is completely degraded, presented on MHC class II or targeted for cross-presentation on MHC class I.[4]

FVIII is a soluble antigen that can be efficiently internalized by APCs *in vitro* and *in vivo*.[5,6] In hemophilia A patients, which have reduced or diminished endogenous FVIII levels in the circulation, replacement therapy with exogenous FVIII can lead to the formation of FVIII-neutralizing antibodies, commonly referred to as "inhibitors". Although it is not clear to what kind of endocytic vesicle FVIII is sorted after endocytosis by APCs, it has been shown that the endocytosis is receptor-mediated[5] and there is also evidence that FVIII is efficiently presented on MHC class II.[5,7] Processing of FVIII by APCs leads to the presentation of multiple different FVIII peptides on MHC class II, derived from different domains present in the molecule.[7] As a result, $CD4^+$ T-cell responses in patients with severe hemophilia A are often polyclonal and directed against multiple domains.[8-10] The proteolytic processing of FVIII for presentation on MHC class II generates a limited number of MHC-promiscuous peptides that can be presented by many different MHC class II molecules, but also generates peptides that are presented in a unique MHC haplotype-specific manner.[7] Processing and presentation of antigenic peptides is, however, not only MHC haplotype dependent, but is also sensitive to changes in proteolytic activity inside APCs. Several cytokines, such as interferon-γ (IFN-γ), IL-1β, IL-4, IL-6 and IL-10 are able to change the expression or activity of endo- or lysosomal proteases involved in proteolytic processing of internalized antigens and MHC-class II-associated invariant chain (Ii).[11-14] IFN-γ exerts several effects on the antigen-presentation capacity of APCs. IFN-γ controls the processing and presentation of internalized antigens by modulating the expression of cathepsins B, D, G, L and S.[11] In addition, IFN-γ induces the expression of Gamma-IFN-inducible-lysosomal thiol reductase (GILT), which in turn influences the generation of active forms of these cathepsins.[14] GILT expression also leads to elevated levels of HLA-DM, which assists in the loading of MHC class II with antigenic peptides. Pro-inflammatory cytokine IL-1β is able to increase the expression of cathepsins B and S in DCs, leading to increased formation of stable peptide-MHC class II complexes.[12] Conversely, the anti-inflammatory cytokine IL-10 exerts the opposite effect.[12]

In this study we investigated whether the presentation of FVIII peptides on MHC class II is subject to factors influencing the basic functions of APCs, such as APC cell type,time, presence of cytokines and route of endocytosis. Instead of monitoring the presentation of FVIII peptides by the activation of FVIII-specific T cells, which is biased to the presentation of only certain FVIII peptides, we used a previously established mass-spectrometry approach to identify peptides bound to MHC class II complexes presented by *in vitro* cultured monocyte-derived DCs (moDCs).[7] Our results indicate that addition of cytokines can modulate the presentation of FVIII peptides by APCs. Additionally, we provide evidence that immune complex formation also has pronounced effects on the presentation of FVIII-derived peptides.

Materials and Methods

Subjects

Blood was drawn from HLA-typed healthy volunteers in accordance with Dutch regulations and following approval from Sanquin Ethical Advisory Board in accordance with the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were isolated from freshly drawn, EDTA anticoagulated blood by separation over a Ficoll-Paque PLUS gradient (d = 1.077, GE Healthcare, Uppsala, Sweden).

Reagents

In this study, the following reagents were used: recombinant human FVIII (Advate) was kindly provided by Dr. B.M. Reipert (Baxter Healthcare Corporation, Vienna, Austria). CD14 microbeads (MACS, Miltenyi Biotech Bergisch Gladbach, Germany), anti-CD80-FITC, anti-CD83-APC, anti-CD86-APC (BD Biosciences, USA) and anti-CD14-PE (Sanquin Reagents, Amsterdam, the Netherlands). Cellgro DC serum-free medium, IL-4, IL-1β, IL-6, TNF-α and GM-CSF were obtained from CellGenix (Freiburg, Germany). LPS and PGE_2 were obtained from Sigma-Aldrich (St. Louis, USA). IFN-γ and IL-10 were obtained from Peprotech (Rock Hill, USA). Hybridomas L243 and IVA-12 (anti-HLA-DR) were obtained from ATCC (Wesel, Germany). Construction of full-length VK34 IgG1 and KM33 IgG1 have been described previously.[15-18] Full-length antibodies were expressed in HEK-293 cells and purified using protein A Sepharose (GE Healthcare, United Kingdom).

FVIII endocytosis by monocyte-derived dendritic cells and macrophages

Monocytes were isolated from the PBMC fraction by positive selection using CD14 microbeads and a magnetic cell separator (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the monocytes isolated was determined by flow cytometry analysis.[19] Monocytes were cultured at a concentration of 0.83×10^6 cells/ml in a 6-well plate (Nunc, Roskilde Denmark) in Cellgro medium supplemented with GM-CSF (1000 IU/ml) and IL-4 (800 IU/ml) for 5 days.[19] For macrophage culture, monocytes were resuspended at $2.5x10^6$ cells/well in 6-well plates in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 ng/ml recombinant human M-CSF. After 5 days of culture, the immature moDCs or macrophages were washed and replated in Cellgro medium supplemented with either GM-CSF and IL-4 or M-CSF at a concentration of 2.5×10^6 cells/ml in a final volume of 2 ml. Cells were incubated with 100 nM FVIII for 5 h prior to induction of maturation. When indicated, cells were cultured with 1000 U/ml IL-6, 10 ng/ml IL-1β or 1000 U/ml IFN-γ during endocytosis and maturation. After 5 h, the immature moDCs or macrophages were maturated using either 1 μg/ml LPS or a cocktail of 10 ng/ml IL-1β, 10 ng/ml TNF-α and 1 μg/ml PGE_2, referred to as 'gold standard'[19] for 24 h in the presence of 1% human serum. The adherent maturated moDCs were detached by 5 minute incubation with phosphate buffered saline (PBS) containing 0.25% trisodiumcitrate and washed before analysis.

Flow cytometric analysis of cell-surface phenotype

For determination of the phenotype, cells were washed with PBS containing 0.5% bovine serum albumin (PBS/0.5%BSA) and incubated with 50 µl 1 µg/ml mAb or appropriate isotype controls diluted in PBS/0.5%BSA for 30 min at 4°C. Cells were washed twice and resuspended in PBS/0.5%BSA. 4',6-diamidino-2-phenylindole (DAPI) was added to the cells before analysis to assess cell viability and exclude dead cells from analysis. Cells were analyzed on an LSRII flow cytometer (Beckton Dickinson, San Jose, USA) and analyzed with Flowjo software version 7.5.5 (Tree Star Inc., Ashland, USA).

Purification of HLA-DR presented peptides

HLA-DR molecules were purified from FVIII-treated, maturated moDCs or macrophages essentially as described previously.[7] Briefly, moDC pellets were lysed using 50 mM Tris, pH 7.0 containing 4% Igepal CA-630 (Sigma, St. Louis, USA). HLA-DR was purified from the detergent-soluble fraction by immuno-affinity chromatography using antibody L243-coupled to CNBr Sepharose 4B (Amersham Biosciences, Buckinghamshire, UK) in the presence of protease inhibitors (Complete Protease Inhibitor Cocktail Tablet, 1 tablet per 50 ml buffer, Roche Diagnostics GmbH, Mannheim, Germany). After washing the Sepharose 5 times with 50 mM Tris-HCl pH 7.0, peptides were eluted with 10% acetic acid for 15 min at 70°C. Finally, peptides were purified from the acetic acid eluate using a C18 ziptip (Millipore, Billerica, USA).

Subcellular fractionation

MoDCs cultures and endocytosis of FVIII were performed as described above. Cells were homogenized using a cell homogenizer (Isobiotec, Heidelberg, Germany) in STE buffer (1.25 M sucrose, 100 mM Tris, 5 mM EDTA in the presence of protease inhibitors (Complete Protease Inhibitor Cocktail Tablet, 1 tablet per 50 ml buffer, Roche Diagnostics GmbH, Mannheim, Germany). Postnuclear supernatants (PNS) were obtained by centrifugation at 150 g for 5 minutes at 4°C. For the sucrose separation, PNS were loaded on a linear 5–50% sucrose gradient (11 ml). Density gradients were established by centrifugation in a Beckmann Optima™ L-100 XP ultracentrifuge (Beckmann Instruments, Palo Alto, CA, USA) equipped with a SW 41 rotor for 90 min at 200 000 g at 4°C. Fractions of 0.5 ml were collected from high sucrose density to low sucrose density and analyzed for relative MHC class II protein content by ELISA. Briefly, Nunc-Maxisorp 96-well plates were coated with anti HLA-DR antibody IVA-12 (5 µg/ml) antibody in carbonate buffer (50 mM NaHCO3 pH 9.8) overnight at 4°C. Fractions were subsequently diluted 5x in PBS/0.1%Tween20/0.3% bovine serum albumin and incubated on the plate for 1 h at 37°C. Bound MHC class II was detected with HRP-labeled antibody L243.

Tryptic digestion of proteins

Where indicated, fractions from sucrose gradient were pooled. Ninety percent of the pools was analyzed for HLA-DR presented peptides as described above and 10% of the pool was analyzed

for total protein content by tryptic digestion. Samples of 50 µL were boiled at 100°C for 5 minutes to break organelles. 6 M Urea (20 µL) was added and incubated at RT for 15 minutes. Ammonium bicarbonate (ABC) was added. Dithiothreitol (DTT) was added to a concentration of 7.7 mM. Samples were incubated for 30 minutes at RT. Iodoacetamide was added to a final concentration of 8.9 mM and samples were incubated for 30 minutes at RT in the dark. Samples were digested with 6.25 ng/ml trypsin overnight at 37°C. Digestion was stopped with 5 µL formic acid. Peptides were purified using a C18 ziptip and analyzed by mass spectrometry.

Peptide identification by mass spectrometry

The identification of peptides was performed essentially as described previously.[7] Shortly, peptides were separated using a reversed-phase C18 column at a flowrate of 100 nl/min with gradient from 0 to 35% (v/v) acetonitrile with 0.1% HAc. Eluted peptides were sprayed directly into the LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific Inc, Bremen, Germany) using a nanoelectrospray source with a spray voltage of 1.9 kV. Collision induced dissociation in the ion-trap (35% normalized collision energy) for the five most intensive precursor ions selected from each full scan in the Orbitrap (300-2000 m/z, resolving power 30.000) was performed. To obtain a high mass accuracy, the LTQ Orbitrap was calibrated on a monthly basis using a calibration solution consisting of caffeine, MRFA and Ultramark 1621 as recommended by the manufacturer.

Peptides were identified using a Sequest search algorithm against UniprotKB non-redundant protein database 25.H_sapiens.fasta (53,784 non-redundant entries actually searched), utilizing Proteome Discoverer release version 1.1 software (Thermo Scientific, Bremen, Germany).[20] For trypsin-digested proteins, which were treated with iodoacetamide, searches were performed with a static carbamidomethyl modification. Identification of peptides was performed using the following filter settings: during the Sequest search, we allowed a mass deviation of 20 ppm. Fragment mass tolerance was 0.8 Da.

For semi-quantitative analysis of MHC class II bound peptides by differential expression analysis, duplicate endocytosis experiments were analyzed with SIEVE™ release version 1.2.0 software (Thermo Scientific, Bremen, Germany). This software was used to compare the duplicate experiments and subsequently analyze differences in peptide abundance between samples For peptide identification, the same criteria were used as described above.

Results

The presentation of peptides on MHC class II is time-dependent

Previously, we have established a protocol for analysis of antigen-derived peptide presentation on MHC class II by moDCs.[7] Immunoprecipitation of HLA-DR from moDC cell lysates followed by acid elution enables the identification of HLA-DR-bound peptides by mass spectrometry. The repertoire of MHC class II-presented proteins comprises of both endogenous DC proteins as well as antigens internalized by DCs. In the previous study, endocytosis of 100 nM FVIII was followed by maturation of moDCs for 24 hours using LPS. Here, we show that presentation of FVIII peptides is optimal between 12 and 24 hours, despite the fact that the expression of maturation markers CD80, CD83, CD86 and MHC class II persisted for longer than 24 hours (Figure 1).

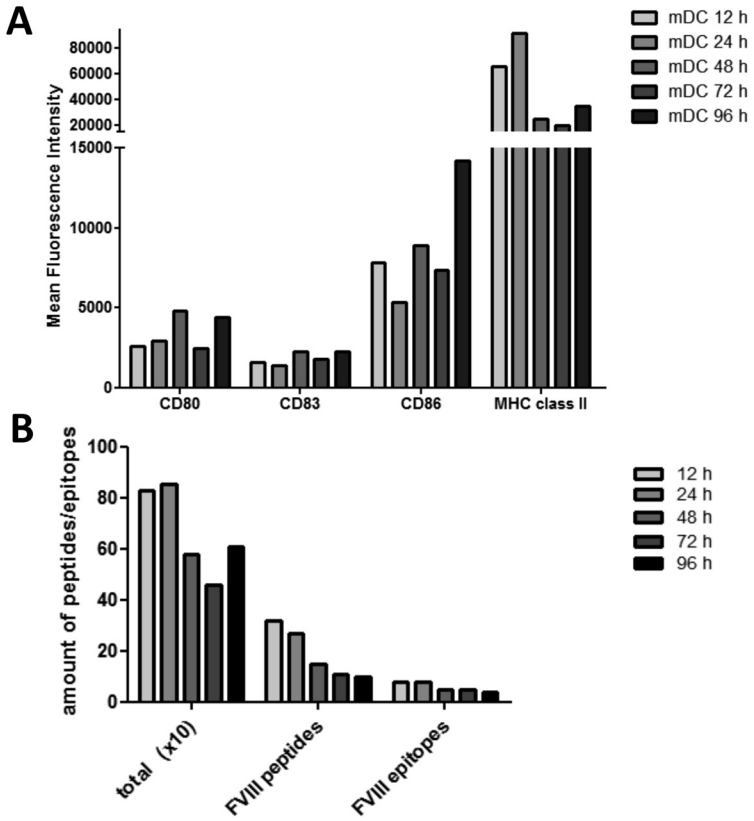

Figure 1. Time-dependent peptide presentation on MHC class II.
Endocytosis of FVIII by immature DCs was followed by maturation with LPS for either 12 h, 24 h, 48 h, 72 h or 96 h. **A)** Cell surface markers CD80, CD83, CD86 and MHC class II were measured by flow cytometry. **B)** Cell lysates were immunoprecipitated with L243-sepharose and analyzed for MHC class II-bound peptides by mass spectrometry. "Total" indicates the total amount of presented peptides.

FVIII-loaded MHC class II is present in immature DCs, but migrates to the plasma membrane after maturation

To gain more insight into the kinetics of FVIII peptide presentation on MHC class II, subcellular fractionations were performed to compare the amount of MHC class II-bound FVIII peptides present inside the cells with those actually presented on the plasma membrane. Per condition, 15 million moDCs were used for this experiment. After endocytosis of FVIII, immature DCs and mature DCs were homogenized and fractionated on a sucrose gradient. Fractions were analyzed for MHC class II content by a sandwich ELISA (Figure 2A). Both immature and mature DCs clearly show 3 distinct peaks of MHC class II-containing fractions. Fractions were pooled accordingly, resulting in a pool 1, 2 and 3. Tryptic digestion of a part of the pools suggests that the most high-density sucrose pool (pool 1) contains mostly intracellular proteins derived from organelles such as mitochondria, MHC class II loading compartments and other vesicles (Figure 2B). The second pool is enriched in plasma membrane and plasma membrane-associated components. The top fractions, which are the lowest in sucrose concentration, contain material from cell organelles that lyzed during the cell disruption procedure. This pool was excluded from subsequent analysis. HLA-DR from the first (intracellular) and second (plasma membrane) pools were subsequently immunoprecipitated and analyzed for the presence of FVIII peptides associated with HLA-DR. Figure 2C shows that immature DCs contain a significant amount of FVIII peptides, which have been processed and loaded on HLA-DR. The amount of these complexes is more or less equal between pool 1, containing 10 FVIII peptides, and pool 2, containing 11 FVIII peptides. This means that approximately half of these peptides is already presented on the plasma membrane. DCs maturated with LPS present a total of 92 FVIII peptides, the majority of which (70) are presented on the plasma membrane.

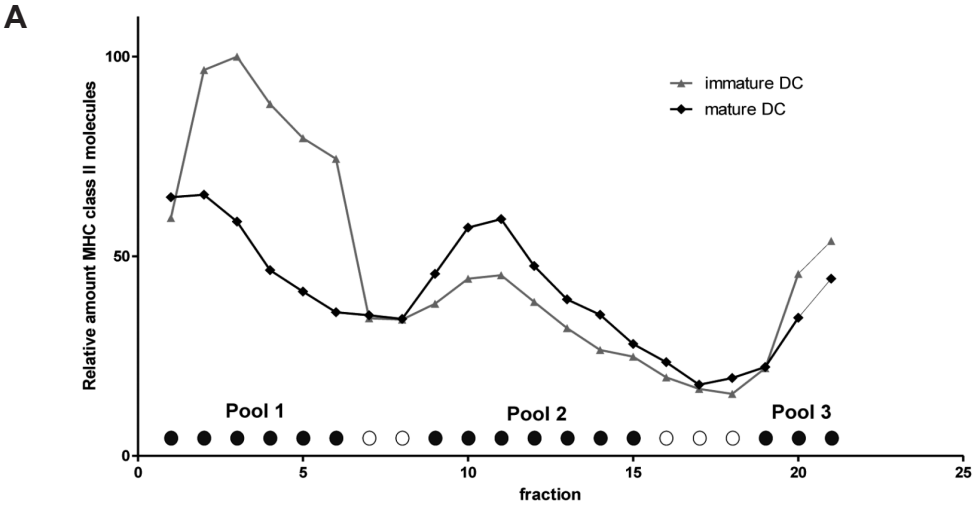

Figure 2. Subcellular localization of FVIII peptide-MHC complexes. Continued on next page.

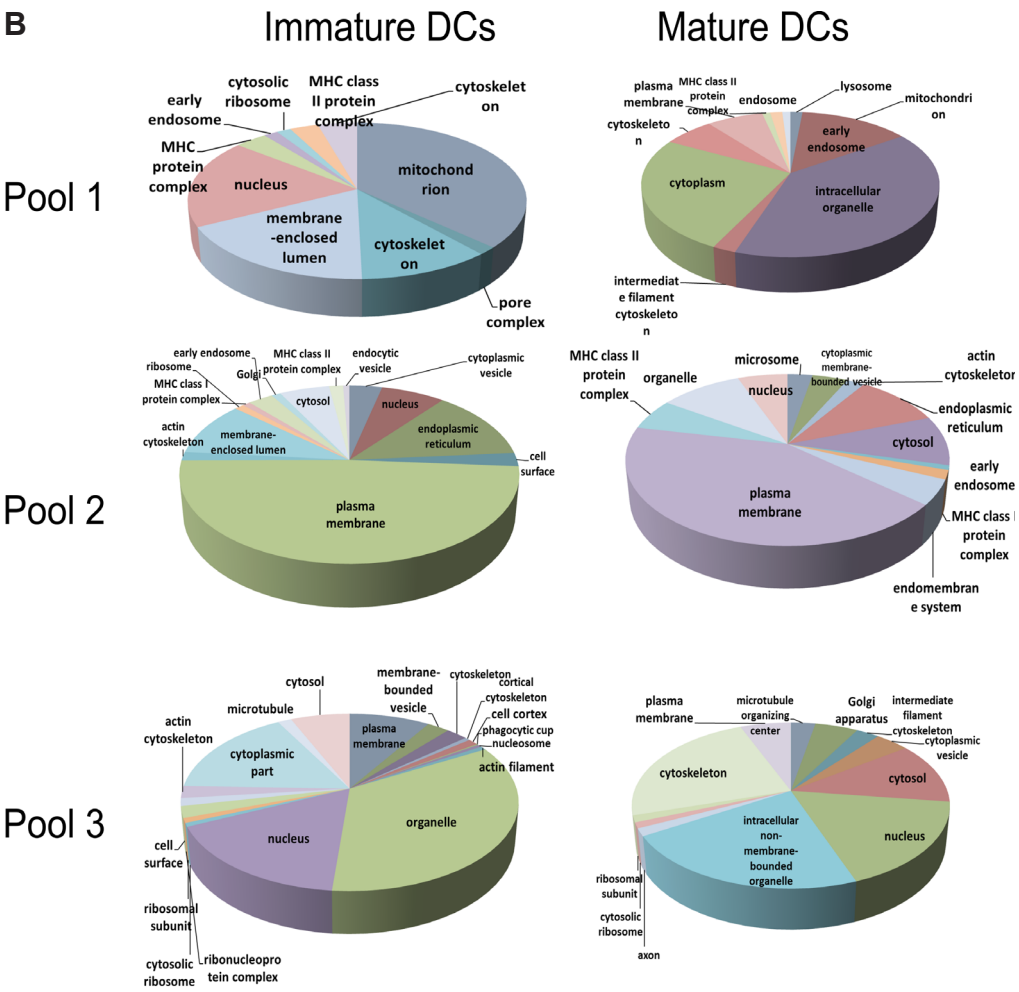

Figure 2. Subcellular localization of FVIII peptide-MHC complexes.
Presentation of FVIII was compared between immature DCs and LPS-atured DCs. Cells were homogenized and PNS was fractionated on a sucrose density-gradient. **A)** Fractions from the sucrose gradient were analyzed by ELISA for MHC class II molecules and divided into 3 pools per sample as indicated. **B)** Peptides from each pool were identified by mass spectrometry after tryptic digestion. Identified proteins were annotated based on subcellular localization or function using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) Bioinformatics Resource. **C)** Pools 1 and 2 from each sample were immunoprecipitated with L243-sepharose and analyzed by mass spectrometry for MHC class II-bound FVIII peptides. IC: intracellular (Pool 1), PM: plasma membrane (pool 2).

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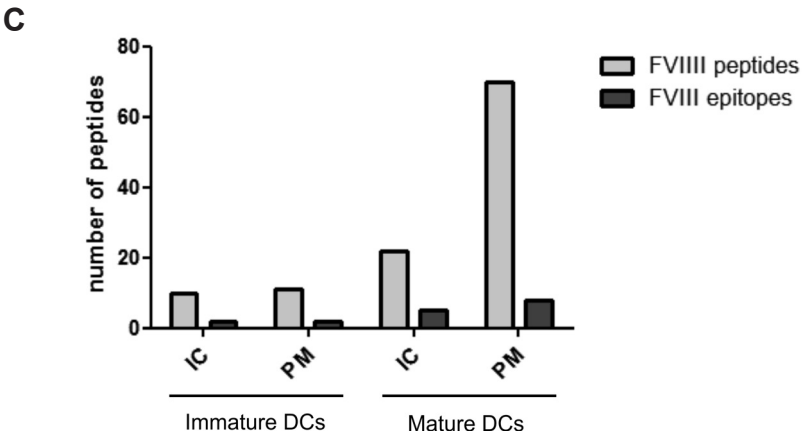

Figure 2. Subcellular localization of FVIII peptide-MHC complexes. Continued from previous page

Dendritic cells are more efficient in FVIII presentation than M2 macrophages

It has been suggested that *in vivo* not dendritic cells, but macrophages are the main APC cell type responsible for endocytosis of FVIII.[6] Therefore, we investigated whether human macrophages are capable of internalizing and presenting FVIII on MHC class II to a similar extent as human DCs. Analysis of the surface markers shows that CD14, which is a receptor present on monocytes and macrophages, but not on DCs, is indeed present only on macrophages. MHC class II is abundantly expressed by both DCs and macrophages. Monocyte-derived macrophages and dendritic cells were able to internalize FVIII to a similar extent, as visualized in Figure 3B. However DCs present FVIII peptides more efficiently than macrophages (Figure 3C). Moreover, macrophages do not only present less FVIII peptides, they also present a smaller variety of FVIII peptides when compared to DCs.

Antibody KM33 inhibits presentation of FVIII-light chain peptides, and to a lesser extent also FVIII-heavy chain peptides

In patients with inhibitors, administration of FVIII leads to the formation of FVIII-antibody immune complexes. To study the recognition and presentation of FVIII-immune complexes, FVIII was pre-incubated with a 25-fold molar excess of either human monoclonal antibody VK34, which is directed against the A2 domain in the heavy chain (HC), or human monoclonal KM33, directed against the C1 domain in the light chain (LC). The complexes were first incubated with moDCs and HLA-DR-presented FVIII peptides were analyzed. As described previously, KM33 inhibits the endocytosis of FVIII by shielding of C1 domain residues which are important for endocytosis of FVIII[18]. In contrast, VK34 does not influence FVIII endocytosis.[18]

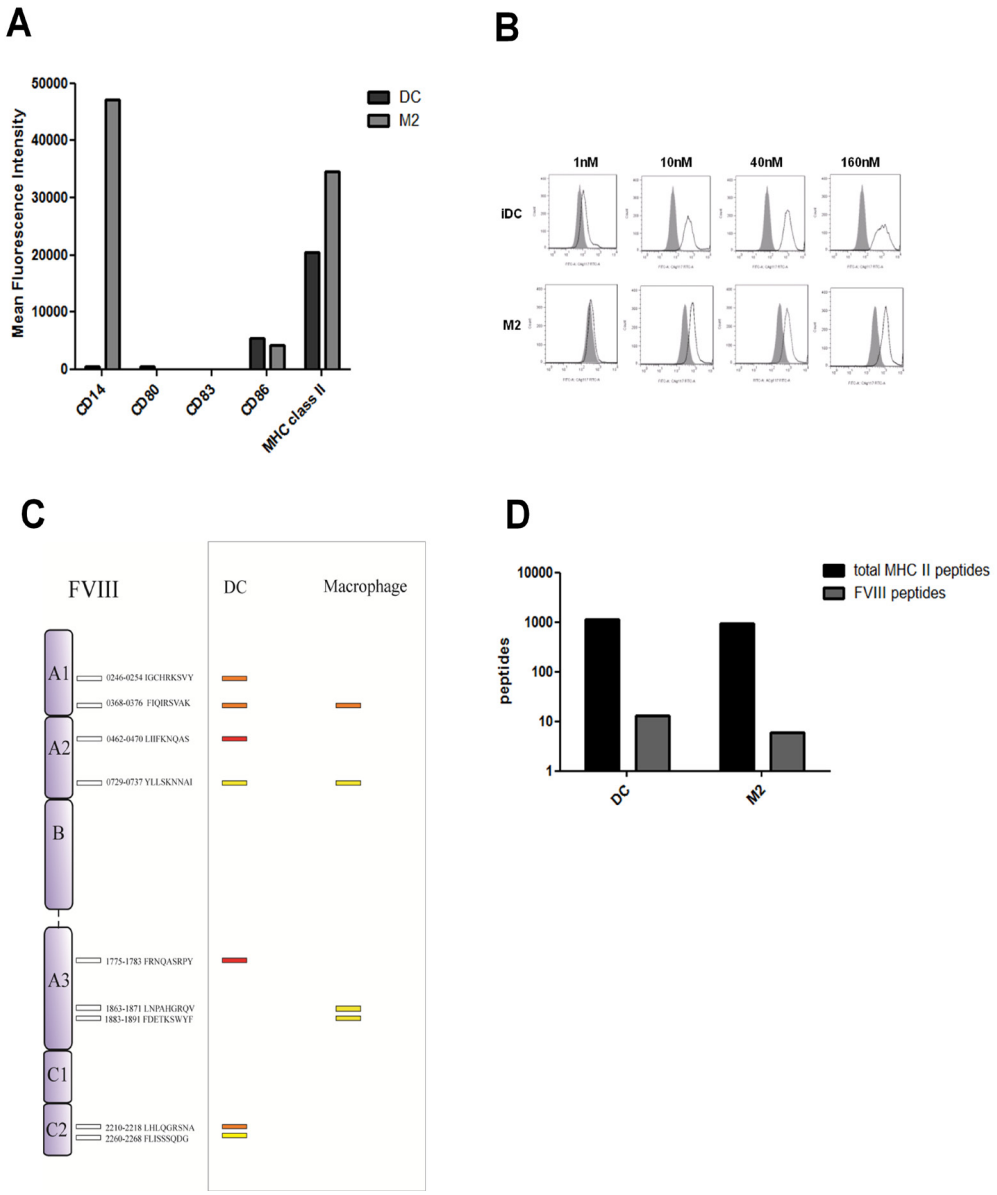

Figure 3. Presentation of FVIII peptides by DCs and macrophages.
Monocytes were differentiated into either DCs or M2 macrophages. **A)** Macrophage marker CD14, maturation markers CD80, CD83 and CD86 and the presence of MHC class II on the cell surface of immature cells were measured by flow cytometry before endocytosis of FVIII. **B)** Endocytosis of different concentrations of FVIII by either DCs or M2 macrophages was measured by flow cytometry. **C)** Identification of MHC class II-bound FVIII regions. **D)** Quantification of individual MHC class II-bound FVIII peptides.

Semi-quantitative comparison of FVIII peptides presented after incubation of moDCs with either KM33-FVIII complex or VK34-FVIII complex reveals that under both conditions FVIII peptides are presented (Figure 4). Interestingly, in the case of KM33-FVIII presentation appears to be less efficient and and with a strong preference for HC peptides (depicted in blue). On the other and,endocytosis of FVIII-VK34 complex leads to a preferential presentation of light chain peptides.

IFN-γ, IL-6 and IL-10 alter the presentation of FVIII peptides

Processing of internalized antigens for presentation on MHC class II is dependent on the proteolytic activity of a variety of proteases inside the antigen-presenting cell. Several proteases have been implicated in the generation of peptides for antigen presentation, such as cathepsins B, E, G, L, S and asparagine-specific endopeptidase (AEP).[11,12,21-23] Several cytokines are able to

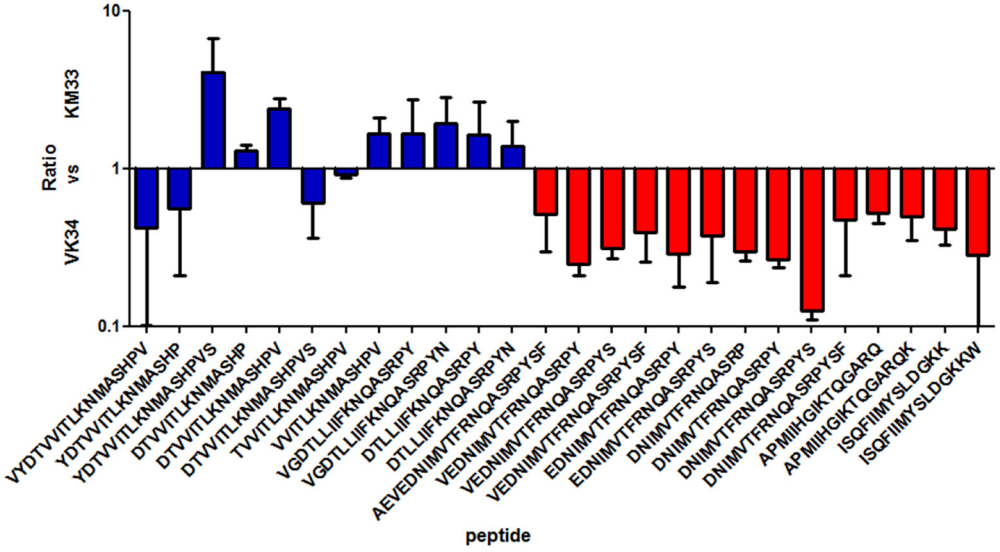

Figure 4. Relative changes in FVIII peptide presentation upon complex formation with monoclonal antibodies.

FVIII was pre-incubated with a molar excess of either antibody VK34 or antibody KM33. FVIII-complexes were added to immature DCs. Following endocytosis, MHC class II-bound FVIII peptides were identified by mass spectrometry. Experiments were performed in duplicate. SIEVE was used to compare intensities of individual peptides between the two conditions. Differences of peptides in relative abundance between the conditions are expressed in a ratio with SD. Ratio >1 means that a peptide is present more abundantly in the FVII-KM33 samples. Ratio <1 means that a peptide is present more abundantly in the FVII-VK34 samples. FVIII-heavy chain peptides are indicated in blue and FVIII-light chain peptides in red.

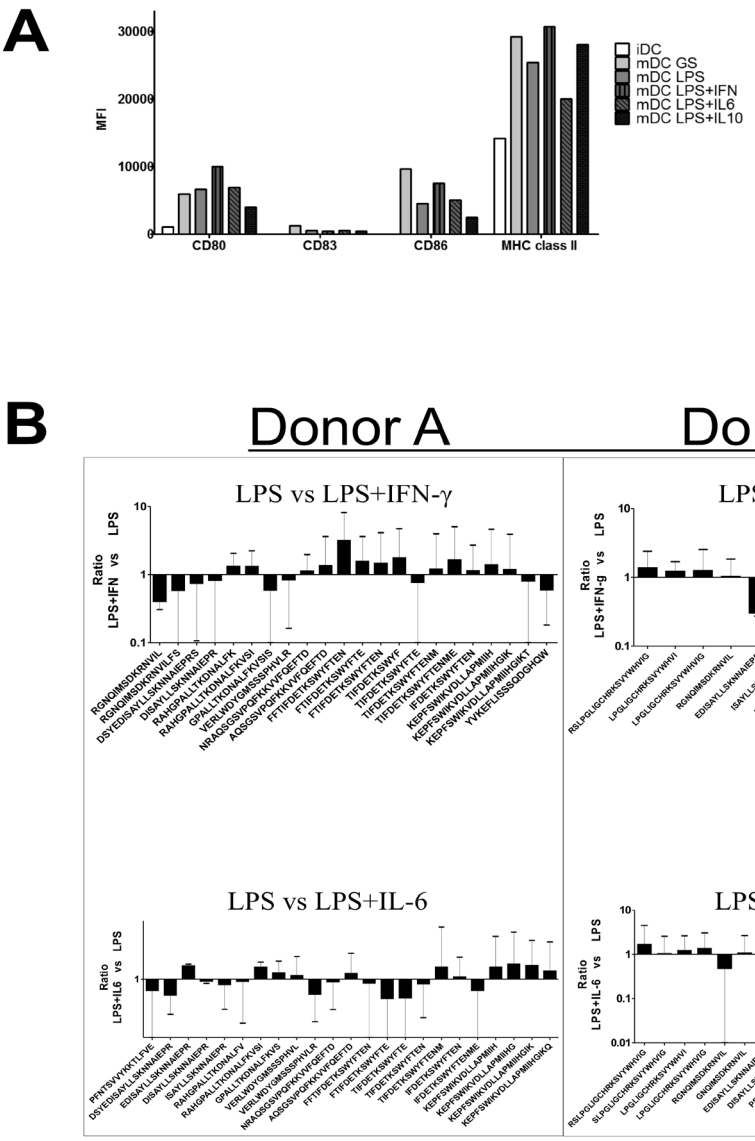

Figure 5. Relative changes in FVIII peptide presentation induced by the presence of IFN-γ or IL-6. Endocytosis of FVIII by immature DCs and subsequent maturation using LPS took place in the presence of either IFN-γ or IL-6 where indicated. Experiments were performed in two unrelated donors. Experiments were performed in duplicate. SIEVE was used to compare intensities of individual FVIII peptides between cells treated with or without IFN-γ (Figure 5A), and between cells treated with or without IL-6 (Figure 5B) Differences of peptides in relative abundance between the conditions are expressed in a ratio. Ratio >1 means that a peptide is present more abundantly in the LPS-maturated samples. Ratio <1 means that a peptide is present more abundantly in the samples maturated with LPS and either IFN-γ or IL-6.

induce changes in the presentation of antigens by changing the expression or activity of these proteases.[11-14] The effect of IL-6, IL-10 and IFN-γ on the presentation of FVIII peptides was studied by addition of these cytokines during endocytosis of FVIII and subsequent maturation of moDCs. In addition, maturation using LPS was compared with a cocktail of the cytokines IL-1β, PGE_2 and TNF-α (gold standard)[19]. DCs maturated with GS induce differentiation of $CD4^+$ T cells to Th1 as well as Th2 cells, whereas LPS-maturated cells induce mostly differentiation towards Th1 cells.[19] The expression of MHC class II on the surface of moDCs is increased upon maturation and is very similar for all conditions (Figure 5B). Maturation in the presence of IFN-γ upregulates the expression of co-stimulatory molecules CD80 and CD86, which makes these cells more capable of activating $CD4^+$ T cells. Addition of IL-10 lowers the expression of CD80 and CD86, while retaining a similar expression of MHC class II. This makes DCs cultured in the presence of IL-10 good candidates for the induction of tolerance.[24] Addition of IFN-γ or IL-6 can induce 2- to 10-fold differences in the presentation of FVIII peptides on HLA-DR (Figure 5B). Interestingly, in some cases these changes are consistent for multiple peptides of the same epitope. In donor A, 4 peptides encompassing the region 2061-2082 KEPFSWIKVDLLAPMIIH are enriched in samples maturated with LPS as compared to LPS in the presence of IL-6. In contrast, almost all peptides encompassing region 726-740 ISAYLLSKNNAIEPR are enriched in samples maturated with LPS and IL-6 for both donor A and donor B. This suggests that some regions are preferentially presented on HLA-DR upon maturation with LPS, whereas other regions are presented more upon addition of IL-6. Similar observations can be made when cells are cultured in the presence of IFN-γ. In this case peptides covering region 726-740 ISAYLLSKNNAIEPR are also less abundantly present when cells are maturated with LPS alone. The most striking difference was observed for region 1881-1891 TIFDETKSWYF (in donor A), where 7 out of 8 peptides are presented less abundantly in the presence of IFN-γ. IL-10 is able to induce 10- to 100-fold differences in the presentation of FVIII peptides, however these changes appear quite random and are not consistent between peptides containing the same region (Figure 6). Nearly all FVIII peptides are more abundantly presented by cells maturated with LPS as compared to cells maturated with GS.

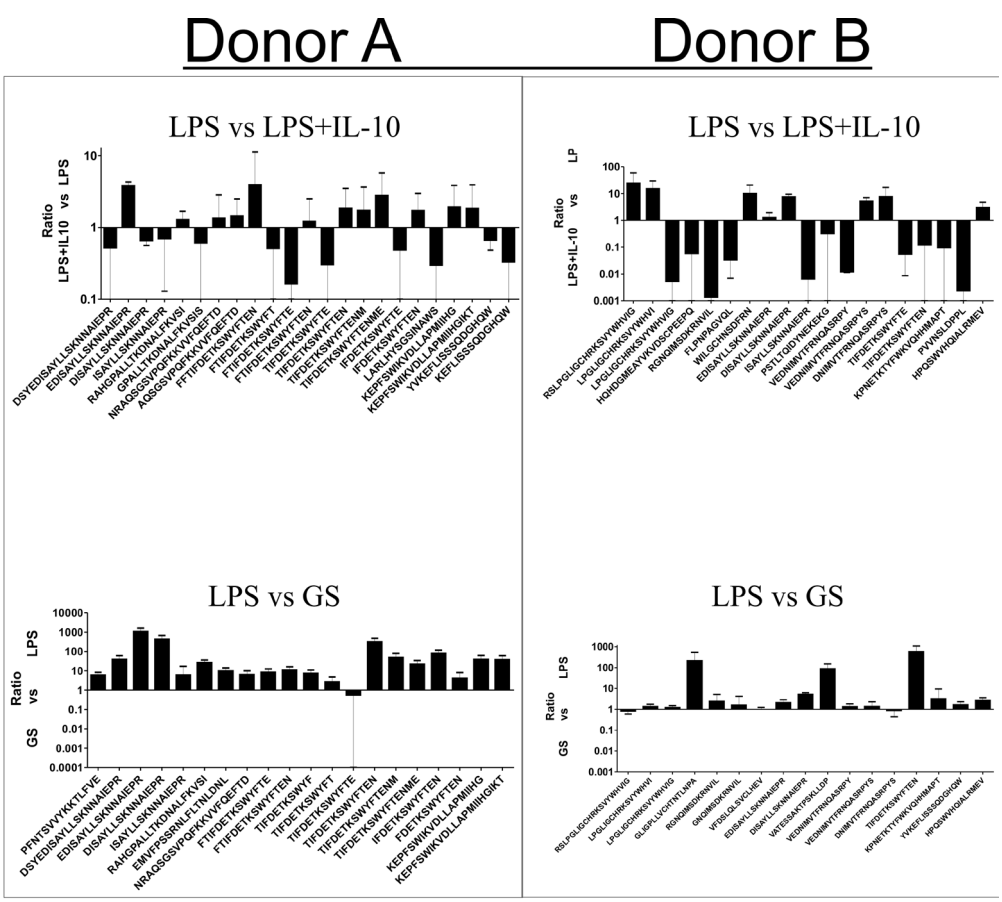

Figure 6. Relative changes in FVIII peptide presentation induced by the presence of IL-10 or maturation with GS.

A) Endocytosis of FVIII by immature DCs and subsequent maturation using LPS took place in the presence or absence of IL-10. B) After endocytosis of FVIII by immature DCs, subsequent maturation was performed using LPS or gold standard (GS). Experiments were performed in two unrelated donors. Experiments were performed in duplicate. SIEVE was used to compare intensities of individual FVIII peptides between cells treated with or without IL-10 (Figure 6A), and between cells maturated with LPS or GS (Figure 6B) Differences of peptides in relative abundance between the conditions are expressed in a ratio. Ratio >1 means that a peptide is present more abundantly in the LPS-maturated samples. Ratio <1 means that a peptide is present more abundantly in the samples maturated with either LPS+IL-10 or with GS.

Discussion

In this study we show that the presence of anti-FVIII antibodies or cytokines appear to induce changes in the preference for presentation of different FVIII peptides. The presentation of FVIII-derived peptides on MHC class II was analyzed by mass spectrometry after endocytosis of FVIII by monocyte-derived APCs.

The presentation of FVIII peptides on HLA-DR peaks between 12 and 24 hours after maturation of moDCs and levels off to about half the amount of peptides when maturation takes place for more than 48 hours, as seen in Figure 1. This amount of FVIII peptides is still presented after 96 hours of maturation. DCs are most likely able to retain this capacity of FVIII presentation because maturation induces a decrease in turnover of MHC class II molecules via downregulation of ubiquitin ligase MARCH1, which tags membrane proteins for endolysosomal degradation.[25,26] Immature DCs are also able to present antigenic peptides on MHC class II, but the turnover of these complexes is much higher, making immature DCs less effective in presentation of antigens to $CD4^+$ T cells.

Immature moDCs do present FVIII peptides, but markedly less than mature moDCs (Figure 2). Cells were fractionated to determine which extent of the FVIII peptides is loaded on HLA-DR inside the cell and which extent is actually loaded on HLA-DR and presented on the plasma membrane. Subcellular fractionation of immature and mature moDCs results in separation of an HLA-DR-positive intracellular fraction, an HLA-DR-positive membrane-enriched fraction and a top fraction containing broken organelles, which was subsequently disregarded. Most FVIII peptides loaded on HLA-DR in mature moDCs are presented on the plasma membrane (Figure 2C). In contrast, HLA-DR molecules loaded with FVIII peptides in immature moDCs are residing both inside the cell and on the plasma membrane. This means that immature DCs are less efficient of presenting FVIII-derived peptides to $CD4^+$ T cells, but are not completely incapable of doing so. The absence of co-stimulatory molecules on immature moDCs (Figure 5a) makes them suitable inducers of immune tolerance towards FVIII. This was already suggested in previous studies, where treatment of hemophilic mice with FVIII-pulsed immature DCs was shown to induce the expansion of regulatory T cells and to inhibit the immune response towards FVIII.[27,28] However,the effects observed in these studies are partial reduction in inhibitor titers. Upon administration of immature DCs *in vivo*, a fraction of the injected cells might mature when given a strong enough stimulus. This could counteract the protective effect of the immature DCs. Therefore, maturation-resistant IL-10-treated DCs have the potential to be more potent inducers of immune tolerance towards FVIII. Further investigation into different APC cell types and their capacity to present FVIII peptides was performed by comparison of dendritic cells and macrophages. *In vivo*, it has been suggested that marginal zone macrophages are the primary cell type responsible for the endocytosis of FVIII in hemophilic mice.[6] Figure 3 shows that monocyte-derived DCs and macrophages are similar in their ability to internalize FVIII, but that DCs are notably more efficient in subsequently presenting FVIII peptides on HLA-DR. Macrophages

present a smaller variety of FVIII peptides. In this experiment seven different FVIII regions were presented by DCs (Figure 3C), compared to only 4 regions that were presented by macrophages.

In order to further dissect the different parameters that can influence the presentation of FVIII by APCs, we compared the presentation of different FVIII peptides on HLA-DR in the presence of different cytokines or in the presence of FVIII-antibody complexes. As shown previously, FVIII endocytosis is almost completely diminished when FVIII is in complex to C1 domain-targeting antibody KM33. When FVIII is in complex with A2 domain-targeting antibody VK34 the endocytosis is unaffected.[18] The binding of these two antibodies also has interesting effects on the presentation of FVIII. Endocytosis of FVIII-KM33 complex results in a reduced presentation of FVIII peptides, as expected, because KM33 inhibits the endocytosis of FVIII. All FVIII peptides that were still presented under this condition, however, are derived from the heavy chain of FVIII. A possible explanation is that the light chain of FVIII is protected from sufficient proteolytic degradation by the interaction with KM33. Supporting this theory, endocytosis of FVIII-VK34 leads to the presentation of almost exclusively light chain peptides, possibly because the heavy chain is protected by its interaction with VK34. Monomeric antigen-antibody complexes have a relatively low affinity for Fc-receptors. Nevertheless, a possible change in the endocytosis route of FVIII-antibody complexes, as compared to FVII alone, due to binding to Fc-receptors cannot be excluded as a possible cause for the observed changes in FVIII presentation. These data suggest that not only endocytosis rate, but also efficacy of proteolytic degradation of FVIII is a crucial step in the presentation of FVIII on HLA-DR.

The cytokine environment plays a prominent role in the regulation of proteolytic activity inside APCs. IL-4 is an established inducer of cysteine cathepsin activity in macrophages[13], TNF-α, IL-6 and IL-1β induce the activity of cathepsins B and S in DCs, whereas IL-10 is able to reduce the activity of these proteases[12,29] and IFN-γ induces the activity of cathepsins B, S and L and of Gamma-IFN-inducible-lysosomal thiol reductase (GILT).[14,30]

The effect of these cytokines on the presentation of FVIII peptides was measured by their addition during endocytosis and/or maturation. Maturation of moDCs with GS results in cells with a high expression of MHC class II and of co-stimulatory molecules CD80 and CD86, but appear to be less capable of presenting FVIII peptides than moDCs maturated with LPS. It should be noted that upon maturation with GS, FVIII peptides are still presented on MHC class II, but the relative abundance of the FVIII peptides was lower than upon maturation with LPS. It appears that both the addition of IFN-γ and of IL-6 are able to induce only minor changes in the presentation of FVIII peptides (Figure 5B). These experiments were carried out with two unrelated donors, both presenting a different spectrum of FVIII regions. This makes it difficult to reach a consensus about the effect of these cytokines on the presentation of any FVIII epitopes. There was no major impact of these cytokines on the generation of FVIII peptides. It appears that the presentation of FVIII is mainly determined by affinity of peptides for particular MHC class II molecules and less by differences in antigen processing. To confirm this statement, additional

experiments are needed to establish whether the activity of endo/lysosomal proteases was actually changed by the addition of these cytokines. IL-10 appears to induce more pronounced changes in the presentation of FVIII peptides. This effect, however, is more random and does not affect any specific region. IL-10-treated DCs are capable of presenting the same FVIII regions as DCs treated only with LPS.

Previously, we have shown that the presentation of FVIII peptides is HLA-specific.[7] Some FVIII peptides are HLA-promiscuous and can be presented by donors with different HLA haplotypes, while other FVIII peptides were HLA-restricted and were therefore donor-specific. Here we show that host HLA-type is not the only factor that determines which FVIII peptides are presented on HLA-DR. The quantity of FVIII presentation is dependent on maturation time, on the presence of anti-FVIII antibodies and on the cytokine microenvironment of the APC. The clinical relevance of these findings is dependent on the repertoire of FVIII-specific $CD4^+$ T cells present in hemophilia A patients, of which only limited data is available. The current findings may contribute to the understanding of inhibitor development and might assist in the search for FVIII-specific T cells in patients.

References

1. Lakadamyali M, Rust MJ, Zhuang X. Ligands for clathrin-mediated endocytosis are differentially sorted into distinct populations of early endosomes. *Cell*. 2006;124(5):997-1009.
2. Trombetta ES, Mellman I. Cell biology of antigen processing in vitro and in vivo. *Annu Rev Immunol*. 2005;23:975-1028.
3. Delamarre L, Pack M, Chang H, Mellman I, Trombetta ES. Differential lysosomal proteolysis in antigen-presenting cells determines antigen fate. *Science*. 2005;307(5715):1630-1634.
4. Burgdorf S, Kautz A, Bohnert V, Knolle PA, Kurts C. Distinct pathways of antigen uptake and intracellular routing in CD4 and CD8 T cell activation. *Science*. 2007;316(5824):612-616.
5. Dasgupta S, Navarrete AM, Bayry J, et al. A role for exposed mannosylations in presentation of human therapeutic self-proteins to CD4+ T lymphocytes. *Proc Natl Acad Sci U S A*. 2007;104(21):8965-8970.
6. Navarrete A, Dasgupta S, Delignat S, et al. Splenic marginal zone antigen presenting cells are critical for the primary allo-immune response to therapeutic factor VIII in hemophilia A. *J Thromb Haemost*. 2009;7(11):1816-1823.
7. van Haren SD, Herczenik E, Ten Brinke A, Mertens K, Voorberg J, Meijer AB. HLA-DR-presented peptide-repertoires derived from human monocyte-derived dendritic cells pulsed with blood coagulation factor VIII. *Mol Cell Proteomics*. 2011;10(6):M110 002246.
8. Reding MT, Okita DK, Diethelm-Okita BM, Anderson TA, Conti-Fine BM. Epitope repertoire of human CD4(+) T cells on the A3 domain of coagulation factor VIII. *J Thromb Haemost*. 2004;2(8):1385-1394.
9. Reding MT, Okita DK, Diethelm-Okita BM, Anderson TA, Conti-Fine BM. Human CD4+ T-cell epitope repertoire on the C2 domain of coagulation factor VIII. *J Thromb Haemost*. 2003;1(8):1777-1784.
10. Reding MT, Wu H, Krampf M, et al. CD4+ T cell response to factor VIII in hemophilia A, acquired hemophilia, and healthy subjects. *Thromb Haemost*. 1999;82(2):509-515.
11. Burster T, Beck A, Poeschel S, et al. Interferon-gamma regulates cathepsin G activity in microglia-derived lysosomes and controls the proteolytic processing of myelin basic protein in vitro. *Immunology*. 2007;121(1):82-93.
12. Fiebiger E, Meraner P, Weber E, et al. Cytokines regulate proteolysis in major histocompatibility complex class II-dependent antigen presentation by dendritic cells. *J Exp Med*. 2001;193(8):881-892.
13. Gocheva V, Wang HW, Gadea BB, et al. IL-4 induces cathepsin protease activity in tumor-associated macrophages to promote cancer growth and invasion. *Genes Dev*. 2010;24(3):241-255.
14. Goldstein OG, Hajiaghamohseni LM, Amria S, Sundaram K, Reddy SV, Haque A. Gamma-IFN-inducible-lysosomal thiol reductase modulates acidic proteases and HLA class II antigen processing in melanoma. *Cancer Immunol Immunother*. 2008;57(10):1461-1470.
15. Boel E, Verlaan S, Poppelier MJ, Westerdaal NA, Van Strijp JA, Logtenberg T. Functional human monoclonal antibodies of all isotypes constructed from phage display library-derived single-chain Fv antibody fragments. *JImmunolMethods*. 2000;239(1-2):153-166.
16. Saeland E, Vidarsson G, Leusen JH, et al. Central role of complement in passive protection by human IgG1 and IgG2 anti-pneumococcal antibodies in mice. *J Immunol*. 2003;170(12):6158-6164.
17. Meems H, van den Biggelaar M, Rondaij M, van der Zwaan C, Mertens K, Meijer AB. C1 domain residues Lys 2092 and Phe 2093 are of major importance for the endocytic uptake of coagulation factor VIII. *Int J Biochem Cell Biol*. 2011.

18. Herczenik E, van Haren SD, Wroblewska A, et al. Uptake of blood coagulation factor VIII by dendritic cells is mediated via its C1 domain. *J Allergy Clin Immunol*. 2011. In Press.
19. Ten Brinke A, Karsten ML, Dieker MC, Zwaginga JJ, van Ham SM. The clinical grade maturation cocktail monophosphoryl lipid A plus IFNgamma generates monocyte-derived dendritic cells with the capacity to migrate and induce Th1 polarization. *Vaccine*. 2007;25(41):7145-7152.
20. Yates JR, 3rd, Eng JK, McCormack AL, Schieltz D. Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein database. *Anal Chem*. 1995;67(8):1426-1436.
21. Beck H, Schwarz G, Schroter CJ, et al. Cathepsin S and an asparagine-specific endoprotease dominate the proteolytic processing of human myelin basic protein in vitro. *Eur J Immunol*. 2001;31(12):3726-3736.
22. Belizaire R, Unanue ER. Targeting proteins to distinct subcellular compartments reveals unique requirements for MHC class I and II presentation. *Proc Natl Acad Sci U S A*. 2009.
23. Burster T, Reich M, Zaidi N, Voelter W, Boehm BO, Kalbacher H. Cathepsin E regulates the presentation of tetanus toxin C-fragment in PMA activated primary human B cells. *Biochem Biophys Res Commun*. 2008;377(4):1299-1303.
24. Gregori S, Tomasoni D, Pacciani V, et al. Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10-dependent ILT4/HLA-G pathway. *Blood*. 2010;116(6):935-944.
25. Wilson NS, El-Sukkari D, Villadangos JA. Dendritic cells constitutively present self antigens in their immature state in vivo and regulate antigen presentation by controlling the rates of MHC class II synthesis and endocytosis. *Blood*. 2004;103(6):2187-2195.
26. Young LJ, Wilson NS, Schnorrer P, et al. Differential MHC class II synthesis and ubiquitination confers distinct antigen-presenting properties on conventional and plasmacytoid dendritic cells. *Nat Immunol*. 2008;9(11):1244-1252.
27. Qadura M, Othman M, Waters B, et al. Reduction of the immune response to factor VIII mediated through tolerogenic factor VIII presentation by immature dendritic cells. *J Thromb Haemost*. 2008;6(12):2095-2104.
28. Ragni MV, Wu W, Liang X, Hsieh CC, Cortese-Hassett A, Lu L. Factor VIII-pulsed dendritic cells reduce anti-factor VIII antibody formation in the hemophilia A mouse model. *Exp Hematol*. 2009;37(6):744-754.
29. Yamaguchi T, Naruishi K, Arai H, Nishimura F, Takashiba S. IL-6/sIL-6R enhances cathepsin B and L production via caveolin-1-mediated JNK-AP-1 pathway in human gingival fibroblasts. *J Cell Physiol*. 2008;217(2):423-432.
30. Lah TT, Hawley M, Rock KL, Goldberg AL. Gamma-interferon causes a selective induction of the lysosomal proteases, cathepsins B and L, in macrophages. *FEBS Lett*. 1995;363(1-2):85-89.

Chapter 7

T-cell responses in two unrelated hemophilia A inhibitor subjects include an epitope at the factor VIII R593C missense site

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Abstract

Background: Development of neutralizing anti-factor VIII (FVIII) antibodies ("inhibitors") is a serious clinical problem in hemophilia A. Increased inhibitor risk has been associated with certain FVIII missense substitutions, including R593C in the A2 domain.

Objectives: The aim of this study was to identify T-cell epitopes in FVIII and characterize T-cell responses in two unrelated hemophilia A subjects sharing F8-R593C and HLA-DRB1*1101 genotypes. We hypothesized that the hemophilic substitution site coincides with an important T-cell epitope.

Patients/Methods: The binding affinities of peptides for recombinant HLA-DR proteins were measured and compared to epitope prediction results. $CD4^+$ T cells were stimulated using peptides and stained with fluorescent, peptide-loaded tetramers.

Results: The inhibitor subjects, but not HLA-matched controls, had high-avidity HLA-DRB1*1101-restricted T-cell responses against $FVIII_{589\text{-}608,}$ which contains- the hemophilic missense site. Antigen-specific T cells secreted Th1 and Th2 cytokines and proliferated in response to FVIII and $FVIII_{592\text{-}603}$. $FVIII_{589\text{-}608}$ bound with physiologically relevant (micromolar) IC50 values to recombinant DR0101, DR1101, and DR1501 proteins.

Conclusions: Hemophilia A patients with R593C missense substitutions and these HLA haplotypes had an increased incidence of inhibitors in our cohorts, supporting a paradigm in which presentation of FVIII epitopes containing the wild-type R593 influences the inhibitor risk in this hemophilia A sub-population.

Introduction

FVIII-neutralizing antibodies ("inhibitors") develop in some hemophilia A (HA) patients who receive factor VIII (FVIII) infusions, resulting in bleeding complications.[1-3] Inhibitors are observed in 25-35% of severe HA patients but also can occur in mild/moderately severe HA.[4,5] Inhibitors have been associated with multiple F8 missense genotypes[6], including F8-R593C.[7-9] Multiple lines of evidence, including sequences/subclasses of inhibitory antibodies[10-13], efficacy of anti-CD40L inhibition[14], and the influence of CD4+ cell counts on antibody titers[15], indicate that inhibitor induction, affinity maturation and antibody class switching involve help from CD4+ T cells. Experimental evidence[16-18] has suggested that T-cell responses in mild/moderately severe HA may be directed against epitopes that contain the wild-type FVIII sequence at the hemophilic mutation site. Several studies have also indicated that B-cell epitopes may include the missense site.[9,19-21] Although T-cell proliferation in response to FVIII protein and peptides has been investigated[22-25], further study is warranted to establish the HLA restriction of T-cell epitopes within FVIII, particularly in the context of specific F8 genotypes. This information could improve estimates of inhibitor risk in defined subpopulations, allowing individualized treatment of high-risk patients by reducing their exposure to wild-type FVIII concentrates, and would motivate the design of less immunogenic versions of FVIII.

In the present study, two unrelated HA subjects with F8-R593C genotype and similar HLA-DR haplotypes were studied to characterize T-cell responses and to identify epitopes within FVIII. The *in vitro* antigenicity of synthetic, overlapping peptides spanning the FVIII-A2, FVIII-C1 and FVIII-C2 domains were evaluated. To test our hypothesis that the hemophilic substitution site coincides with an important T-cell epitope, the binding of peptides containing R593 to various recombinant HLA-DR proteins was evaluated, and the results were correlated with reported inhibitor incidences in F8-R593C patient cohorts. Our findings support a paradigm in which binding and presentation of FVIII epitopes containing the wild-type R593 by several common HLA-DR alleles may influence the relative risk of developing an inhibitor in this HA subpopulation.

Materials and Methods

Subjects and blood samples

Samples from two unrelated HA subjects and from eight *HLA-DRB1*1101*-matched healthy controls were used. Subject 1D (*HLA-DRB1*1101* and *DRB1*1302*), from a Dutch cohort of *F8-R593C* patients, had an initial inhibitor titer of 22 Bethesda units (BU)/mL that declined but persisted for years.[26] Prior to inhibitor development, his baseline FVIII clotting activity (FVIII:C) was 20%; this declined to 1% at peak inhibitor titer, indicating that the inhibitor crossreacted to neutralize his endogenous (hemophilic) FVIII, then increased to 1.4% in subsequent years[26]. He received FVIII to support an operation, which boosted his titer to 2 BU/mL and elicited cross-reactive antibodies against the FVIII A2 domain.[9,27] Subject 41A (*HLA-DRB1*1101* and *DRB1*1303*), from a cohort of American *F8-R593C* patients, also developed an inhibitor after receiving FVIII infusions to support surgery. His baseline FVIII:C was 26%. In the month before and after peak titer (34 BU/mL) his FVIII:C activity ranged from ~1-4%, indicating that the initial inhibitor cross-reacted to neutralize his endogenous (hemophilic) FVIII. He was treated with Rituximab and the titer declined. His most recent titer (2007) was undetectable (<0.5 BU/mL). Neither patient underwent immune tolerance induction. Blood samples from both subjects were collected >6 months after their last FVIII infusion. Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll underlay and either frozen (7% DMSO in serum) or assayed immediately. Research was performed with IRB approval from the University of Washington Human Subjects Committee or the Universiteit van Amsterdam Medical Ethics Committee, with written informed consent.

FVIII peptides and protein

20-mer peptides (with 12-residue overlaps) with sequences (Supplementary Table 1, available online at www.journalth.com) spanning the FVIII A2, C1, and C2 domains were synthesized and verified by mass spectrometry (Mimotopes, Clayton Victoria, Australia; Global Peptide Inc., Ft. Collins, CO; Synpep, Dublin, CA; Anaspec, San Jose, CA). Peptides were dissolved at 10-20 mg/mL in DMSO or DMSO/water. Peptide pools contained equal amounts of 3-7 peptides (10 mg/ml total). Recombinant FVIII was obtained from Pharmacia/Upjohn (manufactured by CSL Behring GmbH).

Peptide-binding predictions and assays

The binding affinities of peptides spanning the FVIII-A2 sequence to the HLA-DR1101 protein were predicted using the ProPred MHC class II binding algorithm (http://www.imtech.res.in/raghava/propred/).[28] This program predicts affinities of peptide sequences for common HLA-DR molecules by evaluating their ability to fit into the canonical 9-residue peptide binding groove that is a feature of the MHC Class II. Every possible 9-mer sequence within FVIII-A2 was analyzed with the algorithm's threshold value set

to list binding scores above 0.8. The predicted set of peptides was further narrowed by excluding sequences with valine at position 1 of the DR1101 binding motif (i.e. the fit of the peptide into the groove), since this residue has been shown to bind weakly in this pocket.[29] Peptides with sequences containing R593 or C593 were evaluated regardless of their scores. Affinities of FVIII peptides for HLA-DR monomers were determined experimentally by competition assays. Recombinant HLA-DR0101, DR0301, DR0401, DR1101, DR1104, or DR1501 proteins were incubated with (1) FVIII peptides at 0.05, 0.1, 0.5, 1, 5, 10, and 50 μM plus (2) biotinylated reference peptides that bound to specific DR proteins with high affinity (Supplementary Table 1). The DR proteins were then immobilized in wells coated with anti-DR capture antibody (L243).[30] After washing, residual bound biotinylated peptide was labeled using europium-conjugated streptavidin (Perkin Elmer) and quantified using a Victor2 D fluorometer (Perkin Elmer). Sigmoidal binding curves were simulated and IC50 values (concentration displacing 50% reference peptide) calculated using SigmaPlot (Systat Software, Inc., San Jose, CA).

HLA-DR Tetramers

HLA-DR1101 tetramers were generated as described.[31] Briefly, biotinylated recombinant DR1101 protein was incubated with pooled or individual peptides at 37°C for 72 h with n-octyl-ß-D-glucopyranoside and Pefabloc (Sigma-Aldrich, St. Louis, MO) and conjugated using R-phycoerythrin (PE) streptavidin (Biosource, Camarillo, CA). Tetramer quality was confirmed by staining a reference T-cell clone (not shown).

Isolation and peptide stimulation of primary CD4+ T cells

T-cell isolation was carried out as described.[17,32] Frozen PBMCs from subject 1D were thawed, washed, and CD4+ T cells were fractionated by notouch isolation (Miltenyi Biotec, Auburn, CA). For subject 41A and HLA-matched control subjects, CD4+ T cells were fractionated from freshly isolated PBMCs. Three million autologous, CD4-depleted PBMCs were plated into 48-well plates for 1hr and then washed, leaving a layer of residual adherent cells behind as APCs. Two million purified CD4+ responder cells were then plated into these wells. Wells were stimulated with 10 μg/ml pooled peptides in T-cell medium (RPMI 1640 with 10% human serum, 1 mM sodium pyruvate, 50 U/ml penicillin and 50 μg/ml streptomycin), supplemented with 40 U/ml IL-2 (Hemagen, Waltham, MD) on day 7, and maintained with medium and IL-2.

Tetramer Guided Epitope Mapping (TGEM)

After two weeks, cells were analyzed with DR1101 tetramers as described.[32,33] For subject 1D and a control subject, 0.75 x 105 cells were incubated with tetramers (labeled with PE) loaded with individual FVIII peptides predicted to bind DR1101[28] (table 1) at 37°C for 1 h, then incubated with anti-CD3-PerCP (BD Biosciences, San Jose, CA), anti-CD4-APC (eBioscience, San Diego, CA), and anti-CD25-FITC (eBioscience) at 4°C for 20 min, and then analyzed on a FACSCalibur (Becton Dickinson, San Jose, CA). For subject 41A and a second HLA-matched

control subject, 0.75 x 105 cells were stained in a similar fashion, using tetramers loaded with peptide pools spanning the A2, C1, and C2 domains of FVIII (Supplementary Table 1). Tetramer-positive responses were decoded using tetramers loaded with individual peptides. To define an objective criterion for positive tetramer staining, CD4+ T cells from six non-hemophilic DR1101 donors were "sham" stimulated using DMSO for two weeks and subsequently stained using a panel of DR1101 tetramers. One tetramer ($FVIII_{381-400}$) gave significantly higher background staining, indicating a peptide-specific effect, while all others had a statistically similar background, allowing calculation of a mean background level (Supplementary Figure 1, available online at www.journalth.com). Our criterion for positive staining was designated as the mean background staining plus 3 times the standard error of the mean: 1.53% for $FVIII_{381-400}$ and 0.46% for all other specificities. The latter is consistent with the cut-off used in previous published studies.[17,18,30-33]

Isolation of T-cell clones and a polyclonal line

For all cultures that demonstrated tetramer-positive staining, FVIII-specific T cells were stained and isolated as described[17] following staining with DR1101-PE tetramers and anti-CD4-FITC (eBioscience). CD4+ tetramer-positive cells were sorted using a FACS Vantage (Becton Dickinson) into 96-well plates containing T-cell medium at one cell per well (to produce clones) or 250 cells per well (to produce a polyclonal line) and expanded by adding 2 μg/ml phytohemagglutinin and 200,000 irradiated PBMCs plus IL-2. Expanded cells were stained with DR1101-PE tetramers and analyzed on a FACSCalibur (Becton Dickinson).

Antigen-specific T-cell proliferation assay

T-cell proliferation was assessed as described.[17,18] Briefly, irradiated PBMCs from an HLA-matched (*DRB1*1101*) non-HA donor were plated at 105 cells/well in 100 μl T-cell medium. Peptides (final concentrations 10, 1, 0.1, and 0 μM) and T cells (104 cells/well) were added in 100 μl T-cell medium and plates were incubated at 37°C. Wells were pulsed with [3H]thymidine (1 μCi/well) after 48 h and cells were harvested 18 h later. [3H]thymidine uptake was measured with a scintillation counter, and stimulation indices (SIs) were calculated as the counts per minute (cpm) of peptide- stimulated cultures divided by the cpm with no peptide added.

Cytokine sandwich ELISAs.

Interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-4 (IL-4), interleukin-10 (IL-10) and interleukin-17A (IL-17A) were measured in supernatants by ELISA. Plates were coated with 100 μl of 2-4 μg/ml cytokine-specific antibody (anti-IFN-γ MD-1, anti TNF-α MAb1, anti-IL-4 8D4-8, anti IL-10 JES3-9D7 and anti-IL17A eBio64CAP17, eBioscience) in coating buffer (eBioscience) overnight at 4°C, washed in PBS with 0.05% Tween 20, blocked with diluent solution (eBioscience) for 1hr at room temperature and washed again. Either cytokine standard (100 μl) (Cell Sciences or eBioscience) or 20-50 μl cell supernatant (plus diluent) was

added to each well, and plates were incubated overnight at 4°C and washed. Biotin-labeled antibody (100 µl at 2 µg/ml) (anti-IFN-γ clone 4S.B3, anti-TNF-α MAb11, anti-IL-4 MP4-25D2, anti-IL-10 JES3-12G8, and anti-IL-17 eBio64DEC17, eBioscience) was added and incubated at room temperature for 1hr. Avidin horseradish peroxidase (eBioscience) was added (1:1000 dilution), incubated at room temperature for 30min and washed. Super Aquablue substrate (100 µl) (eBioscience) was then added and A405 measured using a Bio-Rad 550 reader (Hercules, CA). Cytokine concentrations were calculated from linear standard curves for each cytokine. Th1/Th2 ratios were calculated as: ([IFN-γ] + [TNF-α])/([IL-4] + [IL-10]).

Results

Binding of FVIII peptides to DR1101

The two R593C subjects had the *DRB1*1101* allele in common. An MHC class II binding computer prediction algorithm[28] was used to predict which FVIII-A2 peptides might bind to DR1101. For these predictions a higher score (see Table 1) indicates a greater likelihood that the corresponding peptide is capable of binding. Seventeen synthetic peptides corresponding to sequences with the highest predicted binding scores were then tested to empirically determine their *in vitro* affinities for recombinant DR1101 protein. Observed IC50 values ranged from 0.2 μM to >100 μM, the detection limit. As summarized in Table 1, 8 of the 17 peptides with predicted binding scores above 0.8 bound to DR1101 with an IC50 under 10μM. Notably, $FVIII_{581\text{-}600}$, $FVIII_{589\text{-}608}$, and $FVIII_{589\text{-}608,593C}$, all of which contain the missense site, bound to DR1101 with reasonable affinity as compared with the influenza $HA_{306\text{-}318}$ control peptide (Table 2), whereas $FVIII_{581\text{-}600,593C}$ did not.

	FVIII-A2 peptides	Sequence	IC_{50}*	Predicted DR1101 binding score†
1	429–488	**MAYTDETFKTREAIQHESGI**	**8.9 ± 8**	1.3
2	453–472	**LYGEVGDTLLIIFKNQASRP**	**0.2 ± 0.1**	2.7
3	469–488	**ASRPYNIYPHGITDVRPLYS**	> 100	0.8
4	501–520	**FPILPGEIFKYKWTVTVEDG**	> 100	0.9
5	529–548	**LTRYYSSFVNMERDLASGLI**	**0.2 ± 0.06**	1.9
6	541–560	**RDLASGLIGPLLICYKESVD**	25 ± 24	1.3
7	581–600	**ENRSWYLTENIQRFLPNPAG**	**0.5 ± 0.4**	0.8
8	581–600, 593C	ENRSWYLTENIQCFLPNPAG	> 100	1.5
9	589–608	**ENIQRFLPNPAGVQLEDPEF**	**0.5 ± 0.4**	1.4
10	589–608, 593C	**ENIQCFLPNPAGVQLEDPEF**	**1.5 ± 1.7**	1.4
11	605–624	**DPEFQASNIMHSINGYVFDS**	**8.9 ± 20**	3.2
12	610–629	**ASNIMHSINGYVFDSLQLSV**	> 100	1.0
13	637–656	**WYILSIGAQTDFLSVFFSGY**	**0.3 ± 0.4**	4.3
14	653–672	**FSGYTFKHKMVYEDTLTLFP**	20 ± 47	1.9
15	661–680	KMVYEDTLTLFPFSGETVFM	> 20	1.5
16	677–696	**TVFMSMENPGLWILGCHNSD**	> 100	2.0
17	685–704	PGLWILGCHNSDFRNRGMTA	> 100	2.0

Table 1.FVIII-A2 domain peptides predicted to bind DR1101 with high affinity, using the ProPred algorithm[28].

Peptides subsequently pooled and used to stimulate T cells are in bold font; the three remaining peptides contained predicted MHC Class II binding motifs (the 9-residue sequences predicted to fit into the HLADR1101 binding groove, underlined for each peptide) that were also present in one of the other peptides. Binding scores generated by Propred for all peptides are in the far right column (higher scores indicate stronger predicted affinity). Measured IC50 values under 10 are in bold font. † IC50 values are shown in μM ± the standard error of the mean. A lower IC50 value indicates stronger binding. IC50 >100 indicates no detectable binding in the assay. ‡ The binding score reflects expected binding affinity. Higher scores indicate stronger binding.

Class II protein	Reference peptide* (IC_{50} in μM)	IC_{50}† (μM) $FVIII_{581–600}$	IC_{50}† (μM) $FVIII_{581–600,593C}$	IC_{50}† (μM) $FVIII_{589–608}$	IC_{50}† (μM) $FVIII_{589–608,593C}$
DR0101	$HA_{306–318}$ (0.26)	38 ± 30	50 ± 3	4.2 ± 0.3	8.3 ± 0.7
DR0301	$Myo_{137–148}$ (0.82)	44 ± 7	NB	50 ± 4	NB
DR0401	$HA_{306–318}$ (3.1)	48 ± 7	NB	38 ± 3	NB
DR1101	$HA_{306–318}$ (5.0)	1.1 ± 0.1	NB	1.1 ± 0.1	6.3 ± 0.6
DR1104	$VP16_{34–44}$ (3.1)	9.8 ± 0.8	NB	59 ± 3	NB
DR1501	$MBP_{84–102}$ (0.05)	3.7 ± 0.4	56 ± 4	4.6 ± 0.4	9.8 ± 0.6

Table 2 Binding of peptides to DRB1 proteins

* IC50 indicates the strength of interaction between the class II protein and FVIII peptide compared to a reference peptide (sequences shown in Supplementary Table 1). IC50 values for reference peptides are listed in parentheses. Lower numbers indicate stronger interactions. † Values shown ± standard error of the mean. NB indicates no binding.

T-Cell responses to selected peptides

For inhibitor subject 1D, the number of cryo-preserved cells available for study was only sufficient to test responses to a limited number of peptides. Therefore, peptides that contained predicted FVIII-A2 domain epitopes (Table 1) were utilized to query his T-cell responses. These were divided into two 7-peptide pools, which were then used to stimulate CD4+ T cells from this patient and from a control subject. T cells were cultured for 14 days and then stained using DR1101 tetramers loaded with individual peptides. A clear population of CD4+ T cells was stained by tetramers loaded with $FVIII_{589-608}$ (Figure 1), which bound to DR1101 with high affinity (IC50 =0.5±0.4μM). Weaker positive staining was observed for $FVIII_{429-448}$, $FVIII_{469-488}$, and $FVIII_{581-600}$, which bound to DR1101 with IC50 values of 0.5±0.4μM, 8.9±8μM, and ~100μM. Notably, tetramer staining was negative for CD4+ T cells stimulated by the hemophilic peptide $FVIII_{589-608,593C}$. Attempts to stain T cells from the control subject, using tetramers loaded with each of the 14 peptides containing predicted epitopes (Table 1) yielded negative results (not shown).

Mapping epitopes in the FVIII A2, C1, and C2 domains

CD4+ T cells freshly isolated from subject 41A were stimulated with peptides spanning the FVIII A2, C1 and C2 domains, including two peptides with the R593C substitution (Supplementary Table 1). Cells were cultured and evaluated for responses by staining with fluorescent, peptide-loaded DR1101 tetramers. Representative results are shown in Figure 2A. Tetramer staining was above background for CD4+ cells stimulated with FVIII-A2 peptide pools 1, 2 and 6 and with FVIII-C2 pool 1. Therefore, T cells stimulated with these pools were selected for further analysis (decoding) using tetramers loaded with single peptides that comprised these pools (Figure 2B). T cells stimulated using peptide pool 6 showed positive staining by tetramers loaded with $FVIII_{589-608}$ and $FVIII_{581-600}$, both of which bound with IC50 values of 0.5±0.4 μM.

FVIII-A2 peptide pool 2 and FVIII-C2 peptide pool 1 showed weaker positive staining by tetramers loaded with $FVIII_{421-440}$ and $FVIII_{2187-2205}$ respectively. The IC50 values for these peptides were 5.0±18μM, and 12±26μM. The apparent positive staining of A2 peptide pool 1 was due to $FVIII_{381-400}$, which caused high peptide-specific background staining. Tetramer-stained cells were generally CD25+, suggesting they were activated (not shown). Notably, staining with tetramers loaded with FVIII-A2 peptide pool 11, which contains two peptides with the hemophilic R593C substitution, was negative, indicating that neither peptide containing C593 elicited a high-avidity T-cell response. The same peptide-loaded tetramers were used to evaluate T-cell responses for an *HLA-DRB1*1101* control subject. All staining results using T cells from this subject were negative (not shown).

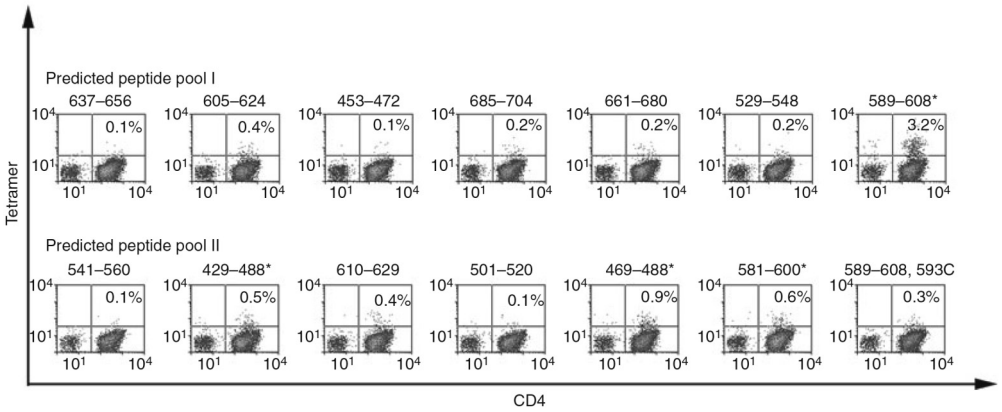

Figure 1. T-cell epitopes recognized by subject 1D.
CD4+ cells were stimulated using two pools of seven FVIII peptides each with predicted *HLA-DRB1*1101*-restricted epitopes. Peptides that elicited a tetramer-positive CD4+ population (greater than three times the standard error of the mean above background) are indicated by asterisks. These included $FVIII_{429-448}$, $FVIII_{469-488}$, $FVIII_{581-600}$, and $FVIII_{581-600}$.

Isolating T-cell Clones and evaluating additional control subjects

To facilitate further study of FVIII-specific T-cell responses, cells from each positive well were stained again and single-cell sorted to obtain FVIII specific T-cell clones and lines (as described in Materials and Methods). Multiple high-affinity FVIII589-608-specific T-cell clones and lines were isolated. Sorted cells with other specificities did not expand. To evaluate the disease specificity of the DR1101-restricted T-cell responses observed in these two inhibitor subjects, T cells from six additional non-HA subjects were stimulated with FVIII peptides and stained with tetramers after two weeks of *in vitro* culture. In all cases, tetramer staining was below the positivity threshold (not shown). Despite the limited number of subjects, the magnitude of $FVIII_{589-608}$-specific tetramer staining observed for hemophilic subjects with inhibitors was significantly higher than for healthy subjects ($p=0.045$). No other tetramer-positive signals were statistically different for patients and controls.

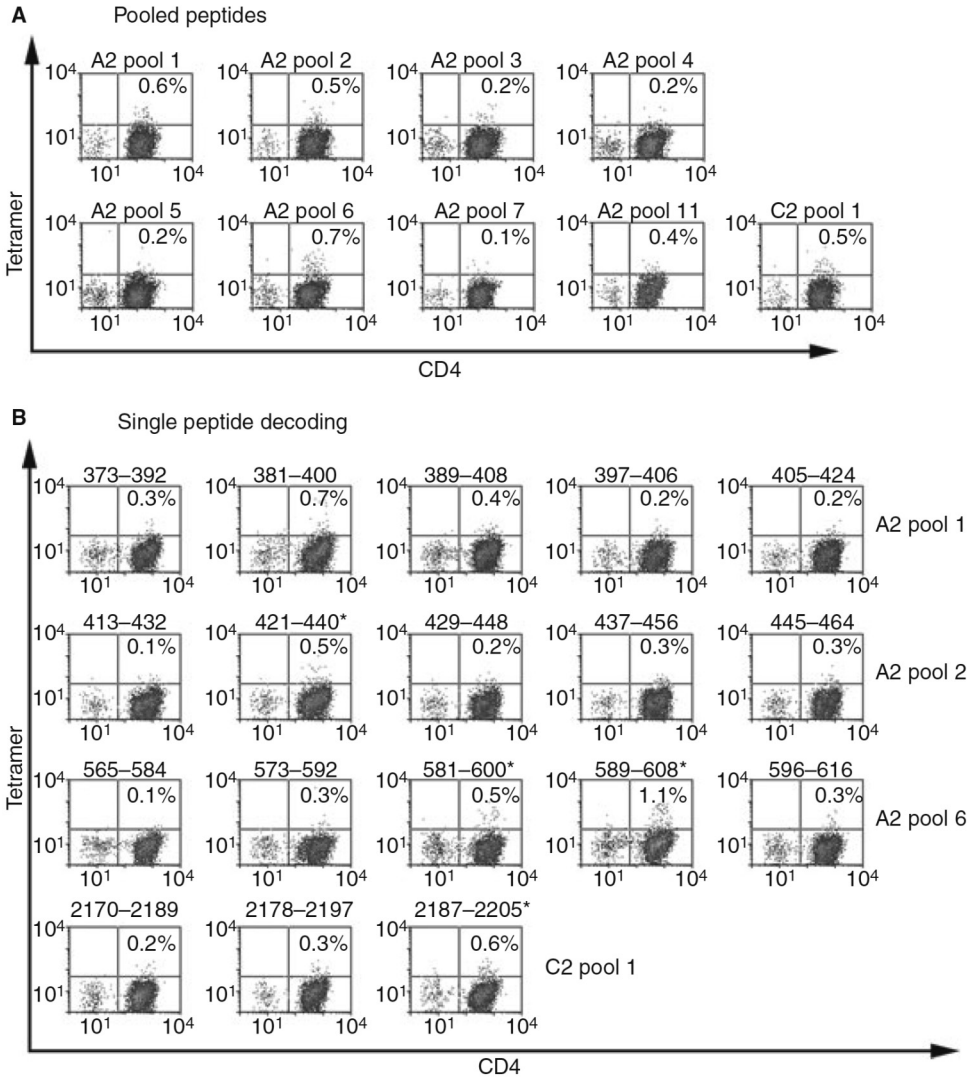

Figure 2. T-cell epitopes recognized by subject 41A.

(A) CD4+ cells were stimulated for two weeks with pooled, overlapping peptides spanning the FVIII A2, C1, and C2 domains. Positive and representative negative tetramer staining results are shown (fluorescent labeling greater than three times the standard error of the mean above background was considered positive). **(B)** Decoding by staining the same cells with HLA-DR1101 tetramers loaded with individual peptides. Peptides that elicited a tetramer-positive CD4+ population are indicated by asterisks. These included $FVIII_{421-440}$, $FVIII_{581-600}$, $FVIII_{581-600}$ and $FVIII_{2187-2205}$ (note that the tetramer loaded with $FVIII_{381-400}$ had an uncharacteristically high background, indicating nonspecific binding to CD4+ cells).

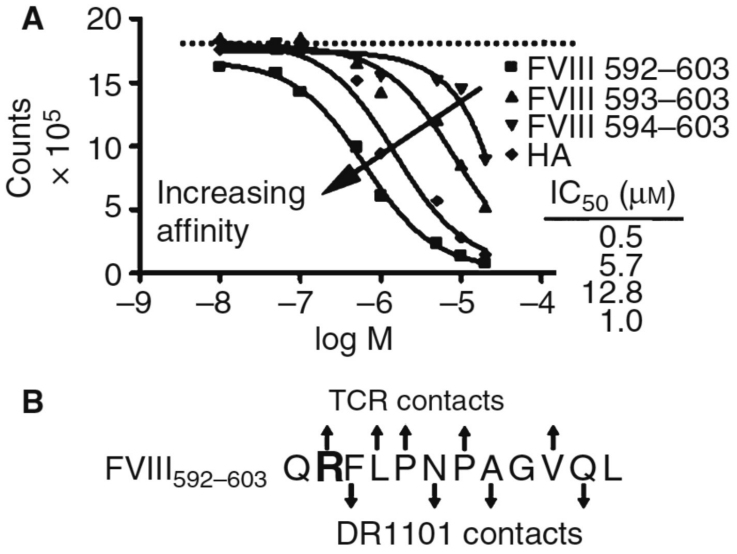

Figure 3. Defining the minimal DR1101-restricted epitope within FVIII589-608.
(A) *In vitro* binding of truncated peptides $FVIII_{592-603}$, $FVIII_{593-603}$ and $FVIII_{594-603}$ and the influenza $HA_{306-318}$ control to HLA-DR1101 protein (arrow indicates increasing affinity). **(B)** Schematic representation of the core HLA-DR1101 binding region within $FVIII_{592-603}$, based on experimental results and the published DR1101 binding motif[29]. Arrows indicate DR1101 contact residues (pointing downward) and possible T-cell receptor contact residues (pointing upward).

Binding of truncated peptides to DR1101

To determine the minimal T-cell epitope within $FVIII_{589-608}$, binding of truncated peptides to recombinant DR1101 was measured in a competition assay (Figure 3A). While $FVIII_{592-603}$ bound with affinity comparable to $FVIII_{589-608}$, the $FVIII_{593-603}$ and $FVIII_{594-603}$ peptides bound with 10-fold and 25-fold lower affinity, respectively. This suggests that residue F594 occupies position 1 of the canonical, nine-residue peptide-binding groove in HLA-DR1101 (Figure 3B), consistent with an epitope predicted by the computer program Propred[28].

T-cell clone proliferation and cytokine secretion

Three antigen-specific T-cell clones and one polyclonal T-cell line were isolated from the same peptide-stimulated cultures used for epitope mapping. Clone 1D-1 was stained by tetramers loaded with $FVIII_{589-608}$ but not with $FVIII_{581-600}$ or an unrelated influenza control peptide, $HA_{306-318}$ (Figure 4A). T cells isolated from subject 41A gave similar results (not shown), indicating that these cells recognize FVIII589-608. Proliferation assays were conducted for these T cells using $FVIII_{589-608}$ and truncated versions of this peptide to determine the functional

epitope. In all cases, residue R593 was essential for maximal proliferation (Figure 4B-E). Interestingly, peptides containing either R593 (wild-type sequence) or C593 (hemophilic sequence) elicited similar proliferation. These T cells proliferated well above background in response to wild-type FVIII protein (Figure 5). Supernatants harvested 48 h following incubation with $FVIII_{589-608}$ were assayed to determine the cytokines secreted in response to FVIII peptide stimulation. Both the T-cell clones and the polyclonal line secreted robust levels of interferon-γ, significant amounts of TNF-α, IL-4, and IL-10, but no IL-17 (Figure 6). Th1/Th2 ratios ranged from 1.8 to 31.6. In the absence of peptidestimulation, cytokine secretion was negligible.

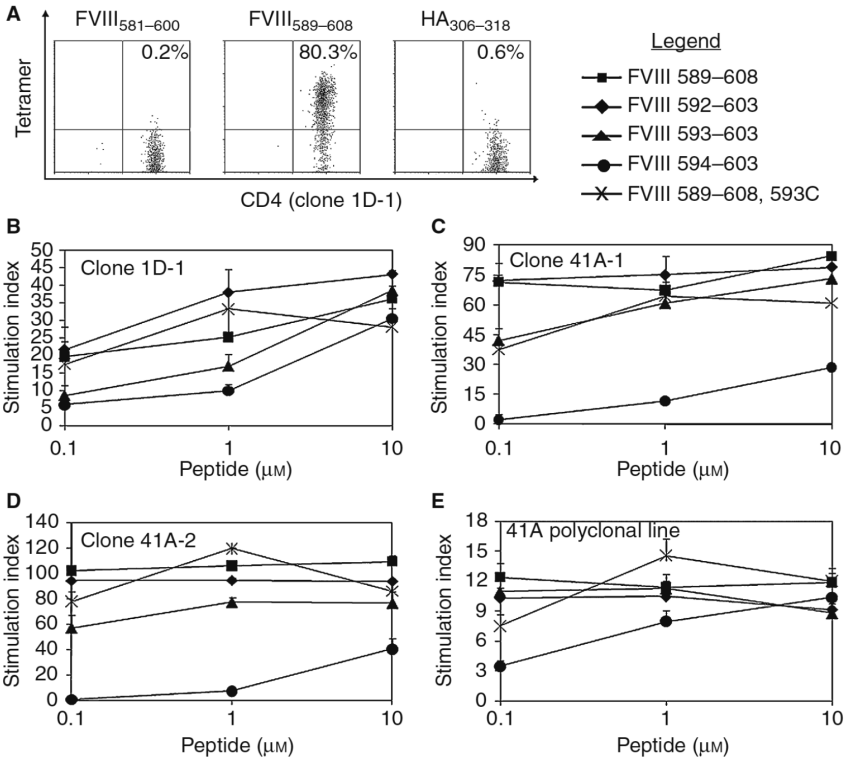

Figure 4. Tetramer staining and proliferation of T-cell clones and a polyclonal T-cell line. (A) Staining of clone 1D-1 using tetramers loaded with $FVIII_{581-600}$, $FVIII_{589-608}$, or the control influenza $HA_{306-318}$ peptide. **(B-E)** Clones from subject 1D (clone 1D-1, B), subject 41A (clones 41A-1 and 41A-2, C-D) and a polyclonal T-cell line from subject 41A (41A Line, E) were stimulated with $FVIII_{589-608}$, $FVIII_{592-603}$, $FVIII_{593-603}$, $FVIII_{594-603}$, and the hemophilic $FVIII_{589-608,593C}$ peptide at 0, 0.1, 1.0, and 10 μM. [3H]thymidine uptake was measured in triplicate wells. Data are expressed as stimulation index values ± standard deviation (SI ± SD), where SI = measured counts / baseline counts.

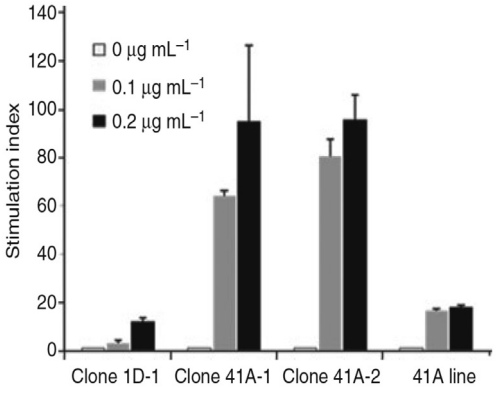

Figure 5. Proliferation of T-cell clones and polyclonal line in response to FVIII.
Clones 1D-1, 41A-1 and 41A-2 and a polyclonal T-cell line from subject 41A were stimulated with 0, 0.1, or 0.2 μg/mL of FVIII protein. [3H]thymidine uptake was measured in triplicate wells (data expressed as SI ± SD).

Binding of FVIII peptides to additional HLA-DR proteins

To determine which common HLA-DR proteins[34] can effectively present FVIII peptides containing the wild-type R593, the binding of $FVIII_{589-608}$, $FVIII_{589-608,593C}$, $FVIII_{581-600}$, and $FVIII_{581-600,593C}$ to DR0101, DR0301, DR0401, DR1101, DR1104 and DR1501 proteins, which represent prevalent HLA-DR haplotypes in the Dutch and American study population, was measured. As summarized in Table 2, $FVIII_{589-608}$ and $FVIII_{589-608,593C}$ bound to DR0101, DR1101 and DR1501. $FVIII_{581-600}$ bound to DR1101, DR1104, and DR1501. These alleles are found in 33% of individuals in European and non-indigenous North American populations[34]. This suggests that a substantial fraction of hemophilia A patients with F8- R593C, those with DRB1*01, DRB1*11, or DRB1*15 haplotypes, may be at increased risk of inhibitor formation. Of course, additional alleles that were not tested in the present study may also be associated with increased inhibitor risk as well.

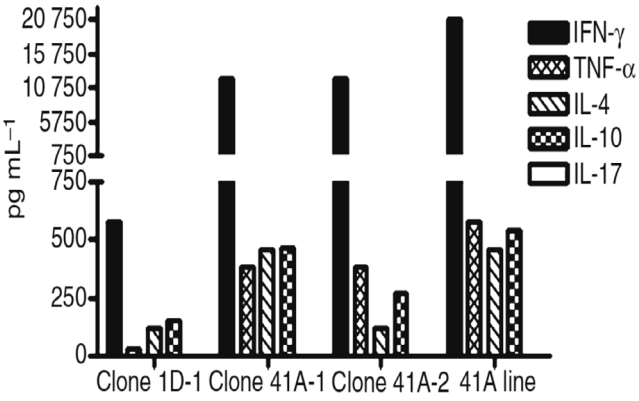

Figure 6. Cytokine secretion by T-cell clones and polyclonal line.
Clones from subject 1D and 41A and a polyclonal T-cell line from subject 41A were stimulated with various concentrations of FVIII589-608 peptide for 48hr. Supernatants were collected and analyzed by ELISA to quantify interferon-γ, TNF-α , IL-4, IL-10 and IL-17 secretion. Cytokines elicited at peptide concentrations of 10 μg/mL are shown, representing averages from triplicate wells.

Discussion

Inhibitory antibodies are the most severe complication affecting HA patients with access to FVIII replacement therapy. However, predicting inhibitor development for individuals remains challenging because risk factors include genetic and environmental components.[35-43] Clinical and experimental evidence suggests that responses to FVIII in mild/moderately severe HA can be triggered by differences between endogenous and infused FVIII and can be potentiated by immune challenges.[17,26]

This study of two unrelated HA subjects with established inhibitors (sharing the *F8-R593C* genotype and *HLADRB1*1101* allele) demonstrated robust T-cell responses directed against an epitope that contains the wild-type FVIII sequence at the hemophilic mutation site. Mild HA patients would only be exposed to this epitope upon treatment or prevention of bleeding episodes by infusions with wild-type FVIII concentrates. Our experiments also showed that the *in vitro* binding affinity of the wild-type FVIII peptide containing R593 for DR1101 was stronger than that of several other peptides containing predicted high-affinity epitopes. In fact, there was only a weak correlation (R2=0.14) between the observed IC50 value and predicted binding score. These results indicate the importance of complementing epitope prediction methods with physical peptide-binding measurements and T-cell assays in order to obtain an accurate assessment of immunogenicity. Many FVIII peptides bound to DR1101 with high affinity but did not elicit T-cell responses, suggesting that both the mild HA subjects and nonhemophilic individuals have central tolerance to these sequences. Some of these sequences may, however, elicit immune responses in severe HA subjects with no circulating FVIII protein.

In agreement with previous studies of mild HA subjects[16,17,44], the experimental results indicate robust T-cell responses directed against an epitope that contains the wild-type sequence at the hemophilic mutation site. For subject 1D (Figure 1), analysis with a limited set of peptides revealed a high affinity T-cell response directed against $FVIII_{589-608}$ and weaker responses directed against an overlapping peptide ($FVIII_{581-600}$) and two distinct sequences ($FVIII_{429-448}$ and $FVIII_{469-488}$) which appeared to be of lower affinity. T-cell responses of subject 41A were queried using a much larger panel of overlapping FVIII peptides that spanned the FVIII A2, C1, and C2 domains (Figure 2), and $FVIII_{589-608}$ again elicited a high affinity response. Weaker, apparently low affinity responses were directed against $FVIII_{421-440}$, $FVIII_{581-600}$ and $FVIII_{2187-2205}$. Expanded $FVIII_{589-608}$-specific T cells from both HA subjects proliferated in response to FVIII protein, indicating that this peptide mimics a naturally processed epitope. Although it is still possible that additional T-cell responses to regions of FVIII not tested here, e.g. the A1, A3 or B domains, may also contribute to FVIII immunogenicity, our results suggest that high affinity *HLA-DRB1*1101*-restricted T-cell responses to an epitope within $FVIII_{589-608}$ contributed to inhibitor formation in both of these HA subjects. Among the peptides that elicited positive responses, only $FVIII_{589-608}$ had significantly higher staining for HA subjects ($p=0.045$) than for healthy control subjects. However, it should be

noted that due to the limited number of HA subjects analyzed, there were insufficient data to conclude that responses to $FVIII_{589-608}$ occur only in hemophilic subjects with inhibitors. In fact, in a previous study of brothers who shared the DR0101 haplotype and had mild HA due to the A2201P missense genotype, both subjects had T-cell responses to the same peptide (which included the mutation site) even though they were discordant for inhibitor development.[18] However, T-cell clones isolated from their blood had distinctly different phenotypes, and IgG concentrated from plasma donated by the "non-inhibitor" brother had a measurable Bethesda titer, indicating he in fact had a circulating but sub-clinical inhibitor.[18,44] Therefore, there is accumulating evidence that T-cell responses such as those characterized here indicate the presence of anti-FVIII antibodies, although actual titers may vary significantly.

T-cell help can drive development and maturation of antibody responses. T cells can also exhibit regulatory phenotypes, including FoxP3 expression, anergy, and IL-10 secretion.[45] Therefore, analysis of tetramer-stained, FVIIIspecific T-cell clones and the polyclonal T-cell line included quantification of representative Th1 and Th2 cytokines, IL-10, and IL-17. FVIII-specific T cells from both inhibitor subjects secreted robust levels of interferon-γ and detectable TNF-α, IL-4, and IL-10, with Th1/Th2 ratios suggesting variable degrees of Th1- polarization. This is consistent with previous observations that interferon-γ and IL-4 are both secreted by FVIII-stimulated CD4+ T cells from inhibitor subjects.[46] A recent study using a HA mouse model suggested that Th1-polarization was associated with tolerance.[47] A study of a mild HA subject[44] showed that *HLA-DRB1*0101*-restricted T-cell clones isolated two years after inhibitor formation were strongly Th2-polarized, while clones isolated at earlier time points secreted interferon-γ and IL-17. Another study of human inhibitor responses concluded that Th2-driven inhibitors occur when the anti-FVIII antibody response is intense, whereas Th1 cells may be involved in the long-term maintenance of anti-FVIII antibody synthesis.[48] Additional studies evaluating changes in T-cell phenotypes and responses over time, particularly in subjects matched by disease severity, genetic characteristics including FVIII genotype and HLA haplotype, and treatment regime, are needed to determine mechanisms leading to tolerance versus high-titer anti-FVIII antibodies.

Initial T-cell proliferation experiments revealed the existence of an epitope within the $FVIII_{589-608}$ peptide. Although responses of the single clone obtained from subject 1D were not as vigorous as those of the cells isolated from subject 41A, proliferation assays indicated robust responses to $FVIII_{592-603}$ for all three clones and for the polyclonal line. Their proliferation was less pronounced in response to $FVIII_{594-603}$, highlighting the importance of the R593 residue. The experimental results and prediction algorithms both indicated that F594 occupies position 1 in the DR1101 peptide-binding groove, while N597, A599 and Q602 fit into the pockets at positions 4, 6 and 9, and adjacent and intervening side chains project outward to interact with T-cell receptors.[49]

Interestingly, all three expanded T-cell clones and the polyclonal line proliferated in response to the hemophilic $FVIII_{589-608,593C}$ peptide, despite the fact that neither primary nor cloned

T cells were stained by tetramers loaded with this peptide, suggesting a lower-avidity interaction of T cells with tetramers or antigen-presenting cells when the hemophilic peptide was presented on the DR1101 surface. Peptide affinities for DR1101 are determined by the fit of peptide "anchor" residues into specific pockets in the class II binding groove, whereas tetramer staining of cells has the additional requirement that the DR1101-peptide complex be recognized by the T-cell receptor on the surface of the responding T cell. Residue 593 is adjacent to the classic 9-residue class II binding motif, but it clearly contributes to binding affinities. The results imply that although the tetramer loaded with the hemophilic peptide was less effective in staining the T cells (so that labeled cells were below the threshold for a "tetramer-positive" response) this lower-avidity interaction was nevertheless strong enough to stimulate T-cell proliferation. This raises the possibility that T cells initially activated by wild-type FVIII can cross-react with wild-type and hemophilic FVIII. This cross-reactivity at the T-cell level may be analogous with cross-reactivity seen at the B-cell level for both subjects, whose inhibitors neutralized their endogenous FVIII. Cross-maintenance of $FVIII_{589-608}$-specific T cells by the endogenous peptide/protein containing the substitution R593C may also contribute to the persistence of immune responses to FVIII; indeed, inhibitors and epitope-specific T-cell responses to FVIII have been observed in mild HA subjects even years after their last infusion.[17,44]

Peptide affinities for a series of HLA-DR proteins indicated that DR0101, DR1104, and DR1501, but not DR0301 and DR0401, can present FVIII peptides containing R593. This reinforces previous suggestions that while HLA haplotypes are not a general risk factor for inhibitor development, certain combinations of FVIII genotype and HLA haplotype may confer an increased risk.[7,50] In the American and Dutch cohorts of *F8-R593C* hemophilia subjects (69 total subjects) nine of the ten (90%) inhibitor subjects had *DRB1*01*, *DRB1*11, or DRB1*15* haplotypes, while 26 of the 59 (44%) subjects without inhibitors had these haplotypes[7] (unpublished data). These alleles are found in 33% of individuals in European and non-indigenous North American populations.[34] Fisher's exact probability test indicates that this is a significant increase (p-value= 0.0076) in inhibitor risk for subjects with these alleles, as compared to all other class II HLA types. However, these results should be replicated using larger populations and accounting for confounding factors such as intensity of treatment[9] and genetic determinants such as IL-10[36] and TNF-α[38] polymorphisms, before drawing firm conclusions about HLA-associated inhibitor risks.

T-cell responses to FVIII were characterized for two unrelated individuals in this study. Both demonstrated Th1-polarized responses (with accompanying low-level IL-4 secretion) directed against a common *HLA-DRB1*1101*-restricted epitope, supporting the notion that T-cell responses to epitopes that contain the hemophilic substitution site contribute to inhibitor formation in mild/moderately severe HA. These T cell responses may occur whenever epitopes containing the wild-type sequence at a missense site are bound to and presented by particular DR proteins at the surface of an antigen-presenting cell. Knowledge of HLArestricted T-cell epitopes in FVIII and their binding affinities for HLA-DR and possibly other MHC class II proteins

should improve predictions of inhibitor risk. Only certain MHC class II proteins on the surface of antigen-presenting cells will be capable of effectively presenting particular FVIII peptides. Additionally, the characterization of immunodominant FVIII T-cell epitopes and T-cell responses will facilitate rational modifications of FVIII, e.g. amino acid substitutions at residues shown to be critical for the interaction with particular MHC Class II molecules. Such FVIII sequence modification could prevent effective epitope presentation and subsequent T-cell activation. This approach should be particularly promising to develop less immunogenic proteins to treat bleeding in individuals with HA genotypes that are associated with increased inhibitor risk.

References

1. Hoyer LW. Factor VIII inhibitors. *Curr Opin Hematol*. 1995;2(5):365-371.
2. Bray GL, Gomperts ED, Courter S, et al. A multicenter study of recombinant factor VIII (recombinate): safety, efficacy, and inhibitor risk in previously untreated patients with hemophilia A. The Recombinate Study Group. *Blood*. 1994;83(9):2428-2435.
3. Kreuz W, Ettingshausen CE, Zyschka A, et al. Inhibitor development in previously untreated patients with hemophilia A: a prospective long-term follow-up comparing plasma-derived and recombinant products. *Semin Thromb Hemost*. 2002;28(3):285-290.
4. Hay CR. Factor VIII inhibitors in mild and moderate-severity haemophilia A. *Haemophilia*. 1998;4(4):558-563.
5. d'Oiron R, Pipe SW, Jacquemin M. Mild/moderate haemophilia A: new insights into molecular mechanisms and inhibitor development. *Haemophilia*. 2008;14 Suppl 3:138-146.
6. Oldenburg J, Pavlova A. Genetic risk factors for inhibitors to factors VIII and IX. *Haemophilia*. 2006;12 Suppl 6:15-22.
7. Bril WS, MacLean PE, Kaijen PH, et al. HLA class II genotype and factor VIII inhibitors in mild haemophilia A patients with an Arg593 to Cys mutation. *Haemophilia*. 2004;10(5):509-514.
8. Thompson AR, Murphy ME, Liu M, et al. Loss of tolerance to exogenous and endogenous factor VIII in a mild hemophilia A patient with an Arg593 to Cys mutation. *Blood*. 1997;90(5):1902-1910.
9. Eckhardt CL, Menke LA, van Ommen CH, et al. Intensive peri-operative use of factor VIII and the Arg593-->Cys mutation are risk factors for inhibitor development in mild/moderate hemophilia A. *J Thromb Haemost*. 2009;7(6):930-937.
10. van den Brink EN, Turenhout EA, Davies J, et al. Human antibodies with specificity for the C2 domain of factor VIII are derived from VH1 germline genes. *Blood*. 2000;95(2):558-563.
11. Fulcher CA, de Graaf MS, Zimmerman TS. FVIII inhibitor IgG subclass and FVIII polypeptide specificity determined by immunoblotting. *Blood*. 1987;69(5):1475-1480.
12. Shima M. Characterization of factor VIII inhibitors. *Int J Hematol*. 2006;83(2):109-118.
13. Lacroix-Desmazes S, Misra N, Bayry J, Mohanty D, Kaveri SV, Kazatchkine MD. Autoantibodies to factor VIII. *Autoimmun Rev*. 2002;1(1-2):105-110.
14. Ewenstein BM, Hoots WK, Lusher JM, et al. Inhibition of CD40 ligand (CD154) in the treatment of factor VIII inhibitors. *Haematologica*. 2000;85(10 Suppl):35-39.
15. Bray GL, Kroner BL, Arkin S, et al. Loss of high-responder inhibitors in patients with severe hemophilia A and human immunodeficiency virus type 1 infection: a report from the Multi-Center Hemophilia Cohort Study. *Am J Hematol*. 1993;42(4):375-379.
16. Jacquemin M, Vantomme V, Buhot C, et al. CD4+ T-cell clones specific for wild-type factor VIII: a molecular mechanism responsible for a higher incidence of inhibitor formation in mild/moderate hemophilia A. *Blood*. 2003;101(4):1351-1358.
17. James EA, Kwok WW, Ettinger RA, Thompson AR, Pratt KP. T-cell responses over time in a mild hemophilia A inhibitor subject: epitope identification and transient immunogenicity of the corresponding self-peptide. *J Thromb Haemost*. 2007;5(12):2399-2407.
18. Ettinger RA, James EA, Kwok WW, Thompson AR, Pratt KP. HLA-DR-restricted T-cell responses to factor VIII epitopes in a mild haemophilia A family with missense substitution A2201P. *Haemophilia*. 2010;16(102):44-55.

19. Peerlinck K, Jacquemin MG, Arnout J, et al. Antifactor VIII antibody inhibiting allogeneic but not autologous factor VIII in patients with mild hemophilia A. *Blood*. 1999;93(7):2267-2273.
20. d'Oiron R, Lavergne JM, Lavend'homme R, et al. Deletion of alanine 2201 in the FVIII C2 domain results in mild hemophilia A by impairing FVIII binding to VWF and phospholipids and destroys a major FVIII antigenic determinant involved in inhibitor development. *Blood*. 2004;103(1):155-157.
21. Jacquemin M, Benhida A, Peerlinck K, et al. A human antibody directed to the factor VIII C1 domain inhibits factor VIII cofactor activity and binding to von Willebrand factor. *Blood*. 2000;95(1):156-163.
22. Hu GL, Okita DK, Conti-Fine BM. T cell recognition of the A2 domain of coagulation factor VIII in hemophilia patients and healthy subjects. *J Thromb Haemost*. 2004;2(11):1908-1917.
23. Reding MT, Wu H, Krampf M, et al. CD4+ T cell response to factor VIII in hemophilia A, acquired hemophilia, and healthy subjects. *Thromb Haemost*. 1999;82(2):509-515.
24. Jones TD, Phillips WJ, Smith BJ, et al. Identification and removal of a promiscuous CD4+ T cell epitope from the C1 domain of factor VIII. *J Thromb Haemost*. 2005;3(5):991-1000.
25. Reding MT, Wu H, Krampf M, et al. Sensitization of CD4+ T cells to coagulation factor VIII: response in congenital and acquired hemophilia patients and in healthy subjects. *Thromb Haemost*. 2000;84(4):643-652.
26. Fijnvandraat K, Turenhout EA, van den Brink EN, et al. The missense mutation Arg593 --> Cys is related to antibody formation in a patient with mild hemophilia A. *Blood*. 1997;89(12):4371-4377.
27. Bril WS, Turenhout EA, Kaijen PH, et al. Analysis of factor VIII inhibitors in a haemophilia A patient with an Arg593-->Cys mutation using phage display. *Br J Haematol*. 2002;119(2):393-396.
28. Singh H, Raghava GP. ProPred: prediction of HLA-DR binding sites. *Bioinformatics*. 2001;17(12):1236-1237.
29. Verreck FA, van de Poel A, Drijfhout JW, Amons R, Coligan JE, Konig F. Natural peptides isolated from Gly86/Val86-containing variants of HLA-DR1, -DR11, -DR13, and -DR52. *Immunogenetics*. 1996;43(6):392-397.
30. James EA, Moustakas AK, Berger D, Huston L, Papadopoulos GK, Kwok WW. Definition of the peptide binding motif within DRB1*1401 restricted epitopes by peptide competition and structural modeling. *Mol Immunol*. 2008;45(9):2651-2659.
31. Novak EJ, Liu AW, Nepom GT, Kwok WW. MHC class II tetramers identify peptide-specific human CD4(+) T cells proliferating in response to influenza A antigen. *J Clin Invest*. 1999;104(12):R63-67.
32. James EA, Bui J, Berger D, Huston L, Roti M, Kwok WW. Tetramer-guided epitope mapping reveals broad, individualized repertoires of tetanus toxin-specific CD4+ T cells and suggests HLA-based differences in epitope recognition. *Int Immunol*. 2007;19(11):1291-1301.
33. Novak EJ, Liu AW, Gebe JA, et al. Tetramer-guided epitope mapping: rapid identification and characterization of immunodominant CD4+ T cell epitopes from complex antigens. *J Immunol*. 2001;166(11):6665-6670.
34. Meyer D, Singe R, SJ M, et al. Single Locus Polymorphism of Classical HLA genes. In: Hansen J ed. Proceedings of the 13th International Histocompatibility Workshop and Conference. Seattle, WA: IHWG Press; 2007:653-704.
35. Oldenburg J, Schroder J, Brackmann HH, Muller-Reible C, Schwaab R, Tuddenham E. Environmental and genetic factors influencing inhibitor development. *Semin Hematol*. 2004;41(1 Suppl 1):82-88.

36. Astermark J, Oldenburg J, Pavlova A, Berntorp E, Lefvert AK. Polymorphisms in the IL10 but not in the IL1beta and IL4 genes are associated with inhibitor development in patients with hemophilia A. *Blood*. 2006;107(8):3167-3172.
37. Lee CA, Lillicrap D, Astermark J. Inhibitor development in hemophiliacs: the roles of genetic versus environmental factors. *Semin Thromb Hemost*. 2006;32 Suppl 2:10-14.
38. Astermark J, Oldenburg J, Carlson J, et al. Polymorphisms in the TNFA gene and the risk of inhibitor development in patients with hemophilia A. *Blood*. 2006;108(12):3739-3745.
39. Repesse Y, Slaoui M, Ferrandiz D, et al. Factor VIII (FVIII) gene mutations in 120 patients with hemophilia A: detection of 26 novel mutations and correlation with FVIII inhibitor development. *J Thromb Haemost*. 2007;5(7):1469-1476.
40. Pavlova A, Delev D, Lacroix-Desmazes S, et al. Impact of polymorphisms of the MHC class II, IL-10, TNF-a and CTLA-4 genes on inhibitor development in severe hemophilia A. *J Thromb Haemost*. 2009;7(12):2006-2015.
41. Gouw SC, van den Berg HM. The multifactorial etiology of inhibitor development in hemophilia: genetics and environment. *Semin Thromb Hemost*. 2009;35(8):723-734.
42. Astermark J, Altisent C, Batorova A, et al. Non-genetic risk factors and the development of inhibitors in haemophilia: a comprehensive review and consensus report. *Haemophilia*. 2010;16(5):747-766.
43. Bafunno V, Santacroce R, Chetta M, et al. Polymorphisms in genes involved in autoimmune disease and the risk of FVIII inhibitor development in Italian patients with haemophilia A. *Haemophilia*. 2010;16(3):469-473.
44. Ettinger RA, James EA, Kwok WW, Thompson AR, Pratt KP. Lineages of human T-cell clones, including TH17/TH1 cells, isolated at different stages of anti-factor VIII immune responses. *Blood*. 2009;114(7):1423-1428.
45. Fehervari Z, Sakaguchi S. CD4+ Tregs and immune control. *J Clin Invest*. 2004;114(9):1209-1217.
46. Hu G, Guo D, Key NS, Conti-Fine BM. Cytokine production by CD4+ T cells specific for coagulation factor VIII in healthy subjects and haemophilia A patients. *Thromb Haemost*. 2007;97(5):788-794.
47. Waters B, Qadura M, Burnett E, et al. Anti-CD3 prevents factor VIII inhibitor development in hemophilia A mice by a regulatory CD4+CD25+-dependent mechanism and by shifting cytokine production to favor a Th1 response. *Blood*. 2009;113(1):193-203.
48. Reding MT, Lei S, Lei H, Green D, Gill J, Conti-Fine BM. Distribution of Th1- and Th2-induced anti-factor VIII IgG subclasses in congenital and acquired hemophilia patients. *Thromb Haemost*. 2002;88(4):568-575.
49. Hammer J, Valsasnini P, Tolba K, et al. Promiscuous and allele-specific anchors in HLA-DR-binding peptides. *Cell*. 1993;74(1):197-203.
50. White GC, Kempton CL, Grimsley A, Nielsen B, Roberts HR. Cellular immune responses in hemophilia: why do inhibitors develop in some, but not all hemophiliacs? *JThrombHaemost*. 2005;3(8):1676-1681.

Chapter 8

General discussion

Protein therapeutics are widely used in the clinic for treatment of various conditions. Therapeutic proteins include recombinant cytokines for immune modulation, hormones such as insulin for treatment of diabetes, monoclonal antibodies for the treatment against tumors or autoimmune disorders and blood factors to control haemostasis. A potential side-effect which often complicates treatment with therapeutic proteins is the development of antibodies against the administered protein. Upon administration of a therapeutic protein it is either recognized as a "self" protein or a "non-self" protein by the recipients' immune system. Antibody formation against therapeutic self-proteins can occur, provided that immune tolerance for this protein is overruled. Such could be the case if the protein possesses immunostimulatory properties. Administration of proteins that are completely, or even partly, non-self is more likely to result in the formation of antibodies, since immune tolerance for this protein has not been established in the recipient.

The development of FVIII-neutralizing antibodies (inhibitors) is a severe, sometimes even life-threatening complication in the treatment of hemophilia A patients with intravenously administered FVIII. Hemophilia A is an X-chromosome-linked bleeding disorder caused by a reduction in plasma concentration or dysfunction of coagulation factor VIII (FVIII). Patients can be classified into groups based on residual FVIII activity in the circulation. Severe hemophilia A patients have less than 1% residual FVIII in the circulation, moderate hemophilia A patients between 1% and 5% and patients with FVIII levels between 5% and 25% are classified as mild hemophilia A patients. The risk of inhibitor formation after prophylactic treatment with FVIII is 25% in the severe group, which is higher than in the other groups. A possible explanation is that because of the lower endogenous FVIII levels, FVIII is more likely to be recognized as non-self. However, severe hemophilia A patients generally require more intensive treatment, which also increases the risk of inhibitor formation.[1] Inhibitor formation in patients with mild or mode-rate hemophilia A is much less frequent, but can occur in patients with certain FVIII missense mutations, for instance at positions 593, 2150 or 2201. These groups of patients have endogenous levels of FVIII to which their immune system has established tolerance. Several studies have revealed that B-cell and T-cell responses against the administered FVIII in these patients are directed against a single epitope consisting of the amino acid that is different from the patients' endogenous FVIII.[2-6] In cases where the $CD4^+$ T-cell response against FVIII is directed against a single region, the possibility that this response is restricted to certain MHC class II alleles becomes greater. Previous studies have suggested weak associations between MHC haplotypes and inhibitor formation.[7-9] However, MHC haplotypes often failed to reach a statistically significant correlation with inhibitor formation. This is most likely due to the large diversity in MHC alleles compared to the relatively small amount of inhibitor patients included in the studies. The presentation of FVIII peptides on MHC class II is an essential part of the activation of $CD4^+$ T cells by antigen-presenting cells (APCs). In this thesis we describe the events that take place during recognition of FVIII by APCs and subsequent activation of FVIII-specific T cells. In Chapter 3 we address the mechanism of endocytosis of FVIII by human monocyte-derived

dendritic cells. Endocytosis of FVIII could be inhibited by binding of monoclonal antibody KM33 to the C1 domain of FVIII. The importance of the C1 domain of FVIII in its endocytosis was further investigated in Chapter 4. Substitutions of three residues in the C1 domain, at positions 2090, 2092 and 2093, to alanine residues almost completely diminished the endocytosis of FVIII by dendritic cells (DCs) as well as macrophages. This variant also induced less antibody formation in hemophilic mice than wild-type FVIII did. Human DCs were also used in Chapter 5 to assess which FVIII peptides are presented on MHC class II after endocytosis. The results from this chapter revealed that there are regions in the FVIII molecule that are commonly presented by multiple donors with different HLA haplotypes. Additionally, some regions were presented in a donor-specific manner. The presentation of these FVIII peptides on MHC class II is not only dependent on donor HLA type, but also strongly depends on other factors, such as cell type and the presence of antibodies bound to FVIII, as shown in Chapter 6. In Chapter 7 we used recombinant MHC class II tetramers to identify FVIII-specific T cells in 2 unrelated mild hemophilia A patients. In this chapter our findings are discussed and placed in perspective with respect to recent findings from other studies.

Endocytosis of FVIII

In Chapter 3 we investigated the role of the endocytic receptors Low Density Lipoprotein Receptor-Related Protein (LRP), Macrophage Mannose Receptor (MR) and Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN). Although we show that FVIII is able to bind to each of these receptors, we also showed that there is no contribution of these receptors to the endocytosis of FVIII. SiRNA-mediated knockdown of each of these individual receptors did not lead to a significant reduction in FVIII endocytosis. Moreover, simultaneous knockdown of all three receptors also did not affect the endocytosis of FVIII. This is partly in disagreement with other publications regarding the role of these receptors.[10,11] The role of LRP in the endocytosis of FVIII is quite clear. LRP is crucial for the clearance of FVIII from the circulation[12] and is also expressed on APCs (chapter 3). Both the results from chapter 3 and results published by Dasgupta and co-workers, however, indicate that LRP is not important for the endocytosis of FVIII by APCs.[10] The role of MR remains more controversial. Endocytosis of FVIII by APCs has been proposed to be mediated through its surface exposure of non-sialylated mannose-ending sugar moieties. As described previously, FVIII is a heavily glycosylated molecule[13], with most of the sugar moieties present on FVIII shown to be biantennary core-fucosylated carbohydrates, with sialylated termini. These carbohydrates are usually not recognized by C-type lectins. There are, however, two carbohydrate structures, in the A1 domain and in the C1 domain, which were shown to be non-sialylated oligomannose-type structures. Figure 1 depicts a model of FVIII with the oligomannose carbohydrate structures at positions 429 and 2118. These mannose residues were proposed to engage in an interaction with

MR, leading to internalization of FVIII by APCs and subsequent activation of FVIII-specific $CD4^+$ T cells.[11] Such a direct involvement of MR seems unlikely, given the fact that siRNA-mediated knockdown of MR on DCs does not significantly reduce the endocytosis of FVIII. An indirect role for MR is still possible, since we have shown that FVIII is able to bind to MR (Chapter 3). In fact, an indirect role for DC-SIGN cannot be excluded either. Both receptors are capable of binding to FVIII, albeit with a rather low affinity. Our data do conclude that an indirect involvement of MR or DC-SIGN in the endocytosis of FVIII, although possible, is very unlikely, because in the absence of these receptors FVIII is still internalized. Although the exact mechanism of FVIII endocytosis was not identified in this thesis, we have established that the endocytosis is mediated through the C1 domain of FVIII. Human monoclonal antibody KM33, which binds to the C1 domain of FVIII, was able to diminish the internalization of FVIII by APCs, whereas an antibody directed against the A2 domain, VK34, did not reduce the internalization of FVIII.

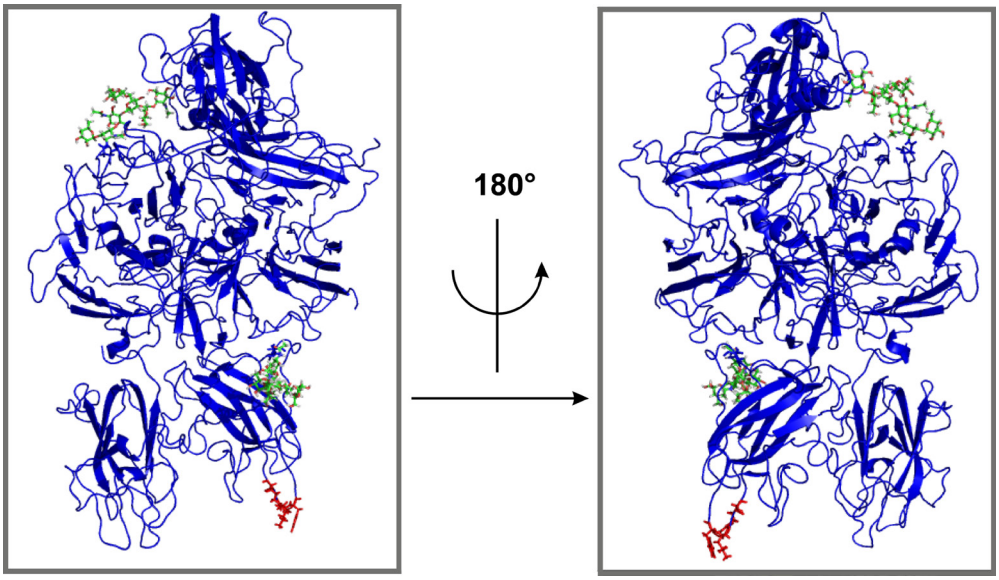

Figure 1. Targets for endocytosis by APCs present in the structure of FVIII.
A representation of the FVIII molecule lacking the B-domain, derived from the molecular structure published by Ngo et al[33]. Two non-sialylated N-linked carbohydrate structures that have been reported to be present at position Asn429 (A1 domain) and Asn2118 (C1 domain)[13] were modeled using the glycam webserver (http://glycam.ccrc.uga.edu/ccrc/). The carbohydrates are depicted as core-fucosylated high mannose structures. The side chains of residues Arg2090, Lys2092 and Phe2093 are depicted in red to indicate that these amino acids are essential for the endocytosis of FVIII.

The inhibitory effect of KM33 can be attributed to the shielding of C1 domain residues Arg2090, Lys2092 and Phe2093. Residues Lys2092 and Phe2093 have previously been established as part of the KM33 epitope.[14] A FVIII variant containing alanine substitutions at these two positions is significantly reduced in endocytosis (Chapter 4), but not to the extent of reduction seen when FVIII is complexed to KM33. An additional alanine substitution at position 2118, the glycosylation site in the C1 domain, did not further reduce the endocytosis of FVIII (data not shown). This implies that the mannose-containing carbohydrate structure at position 2118 is not essential for FVIII endocytosis. The C1 amino acids which were substituted for alanines are indicated in red in Figure 1. This figure shows that the substituted amino acids are in a solvent-exposed loop in the C1 domain, on the opposite side of the C1 domain from the carbohydrate structure at position 2118, which resides at the interface between the A3 domain and the C1 domain. An alanine substitution at position 2090, in addition to the ones at positions 2092 and 2093 showed a further reduced binding to antibody KM33 and further reduced the endocytosis to nearly complete inhibition as well. This triple variant also induced significantly less anti-FVIII antibodies than wild-type FVIII after five subsequent intravenous administrations into hemophilic mice. In summary, the data presented in Chapter 3 and 4 show that a FVIII variant bearing mutations at positions 2090, 2092 and 2093 retains substantial procoagulant activity, but is less immunogenic than wild-type FVIII, by virtue of its reduced endocytosis by APCs.

Presentation of FVIII peptides on MHC class II

An important step in the formation of FVIII inhibitors following FVIII endocytosis by APCs is the presentation of FVIII peptides on MHC class II. APCs are able to activate FVIII-specific $CD4^+$ T cells by surface expression of FVIII peptides on MHC class II in combination with co-stimulatory molecules, such as CD80, CD83 and CD86, and secretion of pro-inflammatory cytokines, such as IL-12. Presentation of FVIII peptides on MHC class II requires FVIII to be proteolytically degraded and targeted to the appropriate intracellular vesicle for loading on MHC class II molecules. Upon endocytosis, internalized antigens, such as FVIII, can be targeted to different endosomal subsets.[15] The endosomal destination determines whether the antigen is completely degraded or presented on MHC class II or cross-presented on MHC class I[16]. Endocytosis of FVIII leads to the presentation of many different FVIII peptides on MHC class II (Chapter 5). Various prediction algorithms are available that can predict which FVIII peptides are capable of fitting in the peptide-binding groove of any particular MHC class II molecule. Prediction of the MHC class II-binding regions present in the complete sequence of FVIII, comprising of 2332 amino acids, results in between 40 and 60 different amino acid regions that are capable of binding to MHC class II, depending on the haplotype analyzed and the algorithm that is used (not shown). These predictions are based entirely on the possibility of a peptide to interact with key amino acids in the β-chain of the MHC molecule

and does not account for other important parameters, e.g., availability of peptides after proteolytic degradation, antigenic competition for available MHC molecules or modifications in the protein such as glycosylation. Therefore, a different method of analyzing the presentation of FVIII peptides is necessary.

In previous studies T-cell responses were analyzed by measuring *in vitro* proliferation of peripheral blood mononuclear cells upon addition of different FVIII peptides.[17-19] These studies are difficult to interpret, since no stimulation threshold was defined for specific stimulation versus aspecific stimulation. The interpretation is particularly difficult because in some cases patients without inhibitors have a stronger T-cell response than patients with inhibitors[18] and in all cases the strongest response was observed in healthy individuals. Moreover, the patients in these studies were not characterized in terms of FVIII mutations or MHC class II haplotype.

In Chapter 5 we have set up a different method to identify potential $CD4^+$ T-cell epitopes within FVIII. We used a mass-spectrometry approach to identify which peptides are presented on MHC class II after endocytosis of FVIII by *in vitro* generated DCs. Figure 2 shows that per donor not 40-60 different FVIII regions are presented, but rather between 10 and 20 different regions. The presentation of FVIII peptides is, as expected, haplotype dependent. Donors with a different MHC haplotype present different FVIII peptides. There are, however, some regions within FVIII which are HLA-promiscuous and can therefore be presented by multiple donors. Examples of such regions are A2 residues 462-470, A3 residues 1775-1783 and C2 residues 2260-2268. Closer inspection of Figures 5 and 6 in Chapter 5 show that these regions are also presented more abundantly than other regions. This suggests that there is a preference for the presentation of HLA-promiscuous peptides, possibly due to the fact that these regions can interact with both MHC class II alleles of the donor. The data from Chapter 5 provide important information regarding the immunogenicity of FVIII and may facilitate the further identification of FVIII-specific $CD4^+$ T cells in hemophilia A patients. In addition, the data from Chapter 5 suggest that donor MHC haplotype is not the only parameter that determines which FVIII peptides are presented. Donors A and D are almost haplo-identical, but present a different repertoire of FVIII peptides. Figure 5 of Chapter 5 also shows that, although the reproducibility of our findings within multiple experiments performed on material from one donor is high, there is some variation in the spectrum of FVIII peptides presented. Therefore, we aimed to identify additional parameters that can influence the presentation of FVIII.

In Chapter 6, we established that, in one donor, *in vitro* generated DCs are more capable of presenting FVIII peptides than *in vitro* generated macrophages, despite the fact that both cell types are equally capable of internalizing FVIII. A possible explanation is that FVIII is targeted to different endosomal subsets after endocytosis by macrophages as compared to DCs. This experiment should be repeated in multiple donors to confirm the relevance of this finding. Not only the amount of presented FVIII peptides was reduced, but the peptides presented by macrophages were derived from different regions than those presented by DCs as well. This finding could have important implications for *in vitro* research

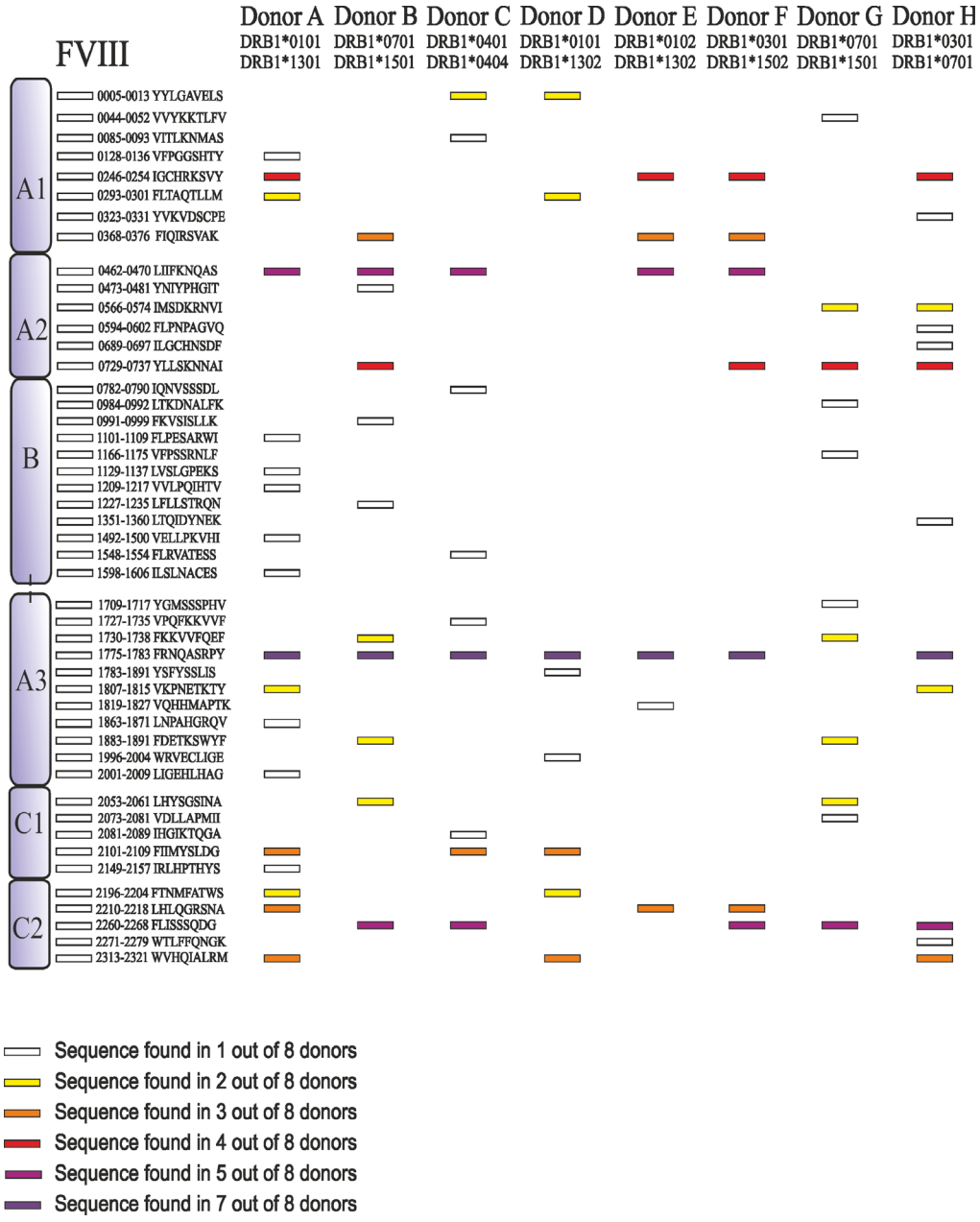

Figure 2. Distribution of FVIII core peptides in eight different donors.
Different core peptides are presented by different donors. FVIII-derived MHC class II peptides are represented as rectangles for each individual donor. The sequences that are common between two or more donors are depicted in color coding as explained in the figure . The different FVIII domains and the location of the peptides are depicted schematically.

regarding inhibitor formation. Most *in vitro* studies investigating FVIII-specific T-cell activation are performed using DCs.[11,20,21] *In vivo* studies, however, have implied splenic macrophages as the most important cell type that internalizes FVIII upon intravenous administration in mice.[22] The monocyte-derived macrophages used in our studies may not be phenotypically similar to the macrophages present in the spleen. It would be interesting to identify FVIII peptides presented *in vivo* by splenic macrophages in order to confirm the relevance of our *in vitro* findings.

Another finding revealed in Chapter 6 is that recognition of FVIII in complex with a monoclonal antibody changes the way in which FVIII is processed intracellularly by DCs, leading to changes in the presentation of FVIII peptides. Human monoclonal antibody KM33 inhibits FVIII endocytosis, as described above. Endocytosis of FVIII-KM33 complex by DCs leads to a significant reduction of the amount of FVIII peptides presented on MHC class II. Interestingly, the remaining FVIII peptides that were presented were all derived from the FVIII heavy chain. In contrast, endocytosis of FVIII in complex with VK34, a human monoclonal antibody directed against the heavy chain which does not reduce FVIII endocytosis, leads to the presentation of almost exclusively FVIII light chain peptides. It appears that the binding of an antibody to either the heavy or the light chain of FVIII reduces the ability of this chain to be presented on MHC class II. A possible explanation could be that the FVIII heavy and light chains dissociate after endocytosis, resulting in different targeting or processing if an antibody is bound to the chain. To test this, FVIII endocytosis should be performed in complex with a larger variety of antibodies and intracellular trafficking of FVIII should be visualized by confocal microscopy.

Presentation of FVIII peptides on MHC class II is not only affected by the presence of an antibody on FVIII, but can also be affected by the presence of certain cytokines. The cytokine environment is an important factor in the regulation of proteolytic activity inside APCs. IL-4, TNF-α, IL-6 and IL-1β induce the activity of cathepsins B and S in DCs and macrophages[23-25], whereas IL-10 is able to reduce the activity of these proteases[23] IFN-γ is in turn able to induce the activity of cathepsins B, S and L and of Gamma-IFN-inducible-lysosomal thiol reductase (GILT).[26,27] The results in Chapter 6 show that some of these cytokines have an effect on the presentation of FVIII peptides by DCs. In particular, IL-10 induces large differences in the efficacy of presentation of many peptides. These differences, however, did not affect any particular region of FVIII. DCs maturated in the presence of LPS and IL-10 are capable of presenting the same FVIII epitopes as DCs treated with LPS alone, which, in combination with their reduced expression of CD80, 83 and 86, makes IL-10-treated DCs an interesting tool for immune tolerance induction.

The results shown in Chapters 5 and 6 were obtained using 8 different donors. Figure 2 summarizes all the different FVIII epitopes presented on MHC class II by these donors. Not all these epitopes will lead to a $CD4^+$ T-cell response in patients with corresponding MHC class II haplotype. This is dependent on the T-cell receptor repertoire present in the patient. Several studies have identified and cloned FVIII-specific $CD4^+$ T cells derived from hemophilia A

patients. Some of the T-cell responses described in those studies were directed against one of the potential FVIII epitopes described in Figure 2.[3,5,6,28,29] The reason that not all peptides from Figure 2 have been shown to elicit a T-cell response is that, in general, the search for T cells against FVIII in hemophilia patients has led to the identification of only a single epitope in each patient. This observation suggests that T-cell responses against FVIII *in vivo* are low in clonal diversity, which is probably incorrect. Most of the T-cell epitopes were identified in mild or moderate hemophilia A patients with a missense mutation. As mentioned earlier, in these patients the T-cell responses are likely to be directed against a single epitope encompassing the area of the mutation. The studies that identify FVIII-specific T cells in mild or moderate hemophilia A patients have so far confirmed this hypothesis.[3,5,28] The T-cell repertoire of severe hemophilia A patients is most probably higher in clonal diversity and therefore also in epitope diversity. The dilemma that one faces when trying to determine the antigen-specific T-cell epitope diversity in a patient is that this diversity is subject to clonal selection by competition.[30,31] As an anti-FVIII immune response progresses, T cells have to compete with each other for available cytokines and antigen. Absence of these factors induces apoptosis[32], so competition for antigen and FVIII leads to loss of diversity. This notion makes it very likely that the repertoire of FVIII-specific T cells in hemophilia A patients is dependent on time after first exposure to exogenous FVIII and amount of re-exposures.

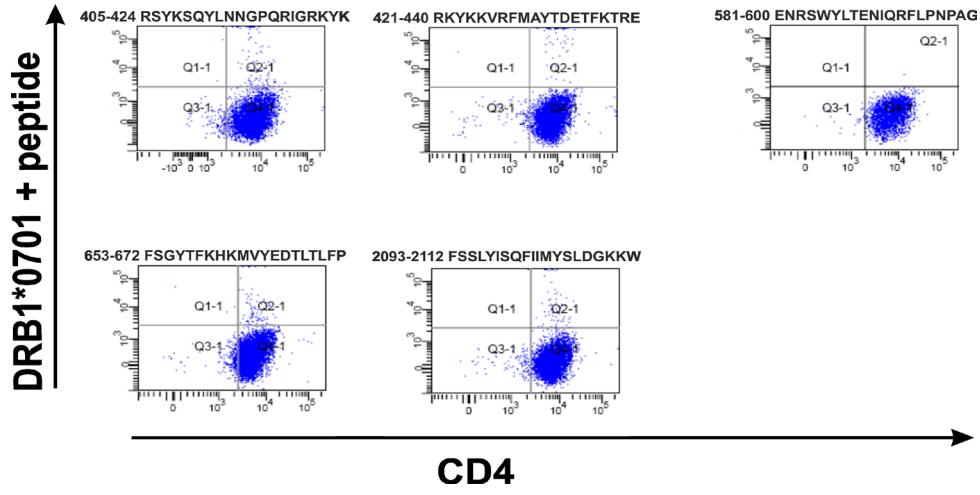

Figure 3. Tetramer analysis of FVIII-specific CD4⁺ T cells in a patient with severe hemophilia
Lymphocytes were stimulated with overlapping synthetic peptides spanning the entire A2, C1 and C2 domain of FVIII. FVIII-specific CD4⁺ T cells were subsequently analyzed by flow cytometry with fluorescent MHC class II tetramers (DRB1*0701) loaded with each individual peptide. The left panel shows the 4 peptides against which FVIII-specific T cells were directed, as indicated by the tetramer-positive population of cells in quadrants Q2-1 (approximately 1% positivity). A representative negative finding is depicted in the right-hand sided panel.

To illustrate the polyclonal nature of an anti-FVIII T-cell response in severe hemophilia A patients, we have used DRB1*0701 tetramers to screen T cells from a severe hemophilia A patient for T cells directed against peptides in the A2, C1 or C2 domain. Overlapping peptides spanning these entire domains were loaded on fluorescent tetramers to identify T cells by flow cytometry. Figure 3 shows that this patient had T cells reactive against 4 different FVIII peptides. A representative negative staining was also shown. Of the four positively identified epitopes in this patient, one epitope corresponds to a peptide identified in Figure 2. It is possible that the other 3 epitopes are specifically presented by DRB1*0701, since none of the donors that have been HLA-typed in Figure 2 have this allele. The T cells used in this assay were derived from blood drawn approximately one year after the peak antibody titer. It would be interesting to investigate whether earlier blood donations contain T cells directed against additional FVIII epitopes to the ones identified here.

Future directions and concluding remarks

Tolerizing strategies to prevent or reduce the occurrence of inhibitors in hemophilia A patients are developing rapidly. Some of these strategies are very expensive, such as immune tolerance induction (ITI) and treatment with bypassing agents. Other potential tolerization strategies in development, such as gene therapy, can be very invasive for the patient. In this thesis, we show evidence that several more cost-effective and less invasive tolerizing strategies are feasible. The creation of FVIII variants with reduced immunogenicity seems the most promising strategy. A FVIII variant was created in Chapter 4 which induced significantly lower anti-FVIII antibody titers than wild-type FVIII. This variant with reduced immunogenicity still induces inhibitor formation *in vivo*, though significantly less than wild-type FVIII. To completely understand why this variant is reduced in its immunogenicity and how to completely abolish inhibitor formation, the exact mechanism of FVIII endocytosis by APCs should be further uncovered, including all the membrane components on the surface of APCs that actually contribute to this process. Further analysis of MHC class II-presented FVIII peptides should include donors that are homozygous for HLA-DR alleles to further dissect the unique properties of each HLA-DR complex for binding to specific FVIII peptides. Analysis of HLA-restrictions for the presentation of FVIII peptides could be used to define and subsequently alter "dangerous" regions within FVIII that are presented in an HLA-promiscuous manner and therefore possess a large potential for $CD4^+$ T-cell activation. The results presented in this thesis could lead to new strategies for intervention of inhibitor formation in patients with hemophilia A.

References

1. Gouw SC, van der Bom JG, Marijke van den Berg H. Treatment-related risk factors of inhibitor development in previously untreated patients with hemophilia A: the CANAL cohort study. *Blood*. 2007;109(11):4648-4654.
2. Bril WS, Turenhout EA, Kaijen PH, et al. Analysis of factor VIII inhibitors in a haemophilia A patient with an Arg593-->Cys mutation using phage display. *Br J Haematol*. 2002;119(2):393-396.
3. James EA, van Haren SD, Ettinger RA, et al. T-cell responses in two unrelated hemophilia A inhibitor subjects include an epitope at the factor VIII R593C missense site. *J Thromb Haemost*. 2011;9(4):689-699.
4. Jacquemin M, Benhida A, Peerlinck K, et al. A human antibody directed to the factor VIII C1 domain inhibits factor VIII cofactor activity and binding to von Willebrand factor. *Blood*. 2000;95(1):156-163.
5. Jacquemin M, Vantomme V, Buhot C, et al. CD4+ T-cell clones specific for wild-type factor VIII: a molecular mechanism responsible for a higher incidence of inhibitor formation in mild/moderate hemophilia A. *Blood*. 2003;101(4):1351-1358.
6. Pratt KP, Qian J, Ellaban E, et al. Immunodominant T-cell epitopes in the factor VIII C2 domain are located within an inhibitory antibody binding site. *Thromb Haemost*. 2004;92(3):522-528.
7. Oldenburg J, Picard JK, Schwaab R, Brackmann HH, Tuddenham EG, Simpson E. HLA genotype of patients with severe haemophilia A due to intron 22 inversion with and without inhibitors of factor VIII. *ThrombHaemost*. 1997;77(2):238-242.
8. Hay CR, Ollier W, Pepper L, et al. HLA class II profile: a weak determinant of factor VIII inhibitor development in severe haemophilia A. UKHCDO Inhibitor Working Party. *Thromb. Haemost*. 1997;77(2):234-237.
9. Bril WS, MacLean PE, Kaijen PH, et al. HLA class II genotype and factor VIII inhibitors in mild haemophilia A patients with an Arg593 to Cys mutation. *Haemophilia*. 2004;10(5):509-514.
10. Dasgupta S, Navarrete AM, Andre S, et al. Factor VIII bypasses CD91/LRP for endocytosis by dendritic cells leading to T-cell activation. *Haematologica*. 2008;93(1):83-89.
11. Dasgupta S, Navarrete AM, Bayry J, et al. A role for exposed mannosylations in presentation of human therapeutic self-proteins to CD4+ T lymphocytes. *Proc Natl Acad Sci U S A*. 2007;104(21):8965-8970.
12. Schwarz HP, Lenting PJ, Binder B, et al. Involvement of low-density lipoprotein receptor-related protein (LRP) in the clearance of factor VIII in von Willebrand factor-deficient mice. *Blood*. 2000;95(5):1703-1708.
13. Medzihradszky KF, Besman MJ, Burlingame AL. Structural characterization of site-specific N-glycosylation of recombinant human factor VIII by reversed-phase high-performance liquid chromatography-electrospray ionization mass spectrometry. *Anal Chem*. 1997;69(19):3986-3994.
14. Meems H, Meijer AB, Cullinan DB, Mertens K, Gilbert GE. Factor VIII C1 domain residues Lys 2092 and Phe 2093 contribute to membrane binding and cofactor activity. *Blood*. 2009;114(18):3938-3946.
15. Lakadamyali M, Rust MJ, Zhuang X. Ligands for clathrin-mediated endocytosis are differentially sorted into distinct populations of early endosomes. *Cell*. 2006;124(5):997-1009.
16. Burgdorf S, Kautz A, Bohnert V, Knolle PA, Kurts C. Distinct pathways of antigen uptake and intracellular routing in CD4 and CD8 T cell activation. *Science*. 2007;316(5824):612-616.

17. Reding MT, Wu H, Krampf M, et al. CD4+ T cell response to factor VIII in hemophilia A, acquired hemophilia, and healthy subjects. *Thromb Haemost*. 1999;82(2):509-515.
18. Reding MT, Okita DK, Diethelm-Okita BM, Anderson TA, Conti-Fine BM. Human CD4+ T-cell epitope repertoire on the C2 domain of coagulation factor VIII. *J Thromb Haemost*. 2003;1(8):1777-1784.
19. Reding MT, Okita DK, Diethelm-Okita BM, Anderson TA, Conti-Fine BM. Epitope repertoire of human CD4(+) T cells on the A3 domain of coagulation factor VIII. *J Thromb Haemost*. 2004;2(8):1385-1394.
20. Dasgupta S, Repesse Y, Bayry J, et al. VWF protects FVIII from endocytosis by dendritic cells and subsequent presentation to immune effectors. *Blood*. 2007;109(2):610-612.
21. Herczenik E, van Haren SD, Wroblewska A, et al. Uptake of blood coagulation factor VIII by dendritic cells is mediated via its C1 domain. *J Allergy Clin Immunol*. 2011. In Press.
22. Navarrete A, Dasgupta S, Delignat S, et al. Splenic marginal zone antigen presenting cells are critical for the primary allo-immune response to therapeutic factor VIII in hemophilia A. *J Thromb Haemost*. 2009;7(11):1816-1823.
23. Fiebiger E, Meraner P, Weber E, et al. Cytokines regulate proteolysis in major histocompatibility complex class II-dependent antigen presentation by dendritic cells. *J Exp Med*. 2001;193(8):881-892.
24. Gocheva V, Wang HW, Gadea BB, et al. IL-4 induces cathepsin protease activity in tumor-associated macrophages to promote cancer growth and invasion. *Genes Dev*. 2010;24(3):241-255.
25. Yamaguchi T, Naruishi K, Arai H, Nishimura F, Takashiba S. IL-6/sIL-6R enhances cathepsin B and L production via caveolin-1-mediated JNK-AP-1 pathway in human gingival fibroblasts. *J Cell Physiol*. 2008;217(2):423-432.
26. Goldstein OG, Hajiaghamohseni LM, Amria S, Sundaram K, Reddy SV, Haque A. Gamma-IFN-inducible-lysosomal thiol reductase modulates acidic proteases and HLA class II antigen processing in melanoma. *Cancer Immunol Immunother*. 2008;57(10):1461-1470.
27. Lah TT, Hawley M, Rock KL, Goldberg AL. Gamma-interferon causes a selective induction of the lysosomal proteases, cathepsins B and L, in macrophages. *FEBS Lett*. 1995;363(1-2):85-89.
28. James EA, Kwok WW, Ettinger RA, Thompson AR, Pratt KP. T-cell responses over time in a mild hemophilia A inhibitor subject: epitope identification and transient immunogenicity of the corresponding self-peptide. *J Thromb Haemost*. 2007;5(12):2399-2407.
29. Ettinger RA, James EA, Kwok WW, Thompson AR, Pratt KP. HLA-DR-restricted T-cell responses to factor VIII epitopes in a mild haemophilia A family with missense substitution A2201P. *Haemophilia*. 2010;16(102):44-55.
30. Fazilleau N, McHeyzer-Williams LJ, McHeyzer-Williams MG. Local development of effector and memory T helper cells. *Curr Opin Immunol*. 2007;19(3):259-267.
31. Busch DH, Pamer EG. T cell affinity maturation by selective expansion during infection. *J Exp Med*. 1999;189(4):701-710.
32. Hughes PD, Belz GT, Fortner KA, Budd RC, Strasser A, Bouillet P. Apoptosis regulators Fas and Bim cooperate in shutdown of chronic immune responses and prevention of autoimmunity. *Immunity*. 2008;28(2):197-205.
33. Ngo JC, Huang M, Roth DA, Furie BC, Furie B. Crystal structure of human factor VIII: implications for the formation of the factor IXa-factor VIIIa complex. *Structure*. 2008;16(4):597-606.

Summary

Hemophilia A is an X chromosome-linked bleeding disorder caused by a reduction or complete absence of coagulation factor VIII (FVIII). The bleeding tendency in patients suffering from hemophilia A can be treated by regular intravenous administrations of FVIII. A severe complication that occurs in approximately 30% of hemophilia A patients is the recognition of administered FVIII as "non-self" by the recipients' immune system, leading to the formation of FVIII-neutralizing antibodies. These antibodies are often referred to as "inhibitors". The formation of inhibitors renders regular prophylactic treatment with FVIII ineffective, due to inactivation of FVIII and rapid clearance from the circulation. Although some treatment-related and genetic determinants have been identified as risk factors for the development of inhibitors, only a limited amount of knowledge is available regarding how and when the patients' immune system deals with administered FVIII, leading to inhibitor formation in one patient and maintenance or establishment of tolerance towards FVIII in another patient.

Inhibitors are mostly high-affinity antibodies of the IgG isotype. The formation of these antibodies requires somatic hypermutation and class-switching of immunoglobulin genes by FVIII-specific B cells, which is controlled by their activation by FVIII-specific $CD4^+$ T cells. The activation of these T cells is in turn dependent on the recognition of FVIII-derived peptides presented on MHC class II by antigen-presenting cells, in combination with recognition of co-stimulatory signals received from the antigen-presenting cell. The aim of the studies presented in this thesis was to dissect the mechanisms of FVIII endocytosis and presentation on MHC class II by antigen-presenting cells, with the intention to provide new targets for modulation of inhibitor formation in hemophilia A patients.

In Chapter 1 general background regarding the formation of inhibitors is presented. This chapter summarizes the current knowledge on the risk factors for inhibitor formation and highlights the basic features of a humoral immune response. The role of antigen-presenting cells (APCs) in the initiation of a humoral response against FVIII is further reviewed in Chapter 2. This chapter describes the current knowledge regarding the endocytosis and presentation of FVIII on MHC class II by APCs.

We have studied the contribution of several endocytic receptors to the endocytosis of FVIII by APC in Chapter 3. Our results show that macrophage mannose receptor (MR), DC-SIGN and Low Density Lipoprotein Receptor-Related Protein (LRP) are able to bind FVIII, but do not significantly contribute to the endocytosis of FVIII by human dendritic cells. We were able to block the endocytosis of FVIII almost completely using human monoclonal anti-FVIII antibody KM33, which is directed against the C1 domain of FVIII. A control antibody directed against the A2 domain of FVIII, VK34, did not block FVIII endocytosis. This implies that KM33 shields a region in the C1 domain of FVIII that mediates its endocytosis by dendritic cells. This result was confirmed *in vivo*, using hemophilic mice. Antibody KM33 was able to protect these mice from inhibitor formation upon repeated infusions of FVIII, whereas the presence of antibody VK34 did not affect inhibitor formation.

Following up on Chapter 3, we pursued the identification of the C1 residues that are shielded

by KM33, to find out whether these residues mediate FVIII endocytosis. In Chapter 4 we have constructed a FVIII variant with alanine substitutions at positions 2090, 2092 and 2093 in the C1 domain of FVIII. This variant, which shows minimal binding to KM33, is internalized significantly less than wild-type FVIII by murine dendritic cells. Repeated infusions of this FVIII variant into hemophilic mice also induces significantly less formation of inhibitors than wild-type FVIII.

The next three chapters address events downstream of FVIII endocytosis in the process of inhibitor formation. These chapters describe the requirements for presentation of FVIII peptides on MHC class II and the recognition of presented FVIII peptides by FVIII-specific $CD4^+$ T cells. In Chapter 5 we introduce a novel assay to identify peptides presented on MHC class II by human monocyte-derived dendritic cells, using mass spectrometry. We were able to show that dendritic cells present peptides derived from endogenous proteins as well as internalized proteins. Endocytosis of FVIII leads to the presentation of FVIII peptides in a donor-dependent manner. Some peptides were presented by multiple donors, while other peptides were presented only by a single donor. This donor-dependent presentation of FVIII peptides can be explained by differences in HLA haplotype. In Chapter 6 we show that environment-related factors are also able to influence the presentation of FVIII peptides. Dendritic cells appear to present FVIII peptides more efficiently than macrophages, despite the fact that these cell types are capable of internalizing FVIII to a similar extent. The presence of antibodies in complex to FVIII also influences the efficacy of FVIII presentation. Complex formation of FVIII with FVIII-heavy chain-directed antibody VK34 induces a preferential presentation of light chain-derived peptides on MHC class II, whereas complex formation with light chain-antibody KM33 results in the presentation of exclusively heavy chain-derived peptides. The presence of several cytokines such as IFN-γ, IL-6 and IL-10 during the maturation of dendritic cells also leads to alterations in the preferences for presentation of FVIII peptides.

In Chapter 7 we used recombinant MHC class II tetramers to identify FVIII-specific $CD4^+$ T cells in two unrelated mild hemophilia A patients with FVIII missense genotype Arg593Cys. Both patients in this study had a DRB1*1101-restricted T-cell response to a FVIII peptide consisting of residues 589-608, which contains the hemophilic missense site. These results are consistent with earlier findings that $CD4^+$ T cells in mild hemophilia A patients are directed against a region consisting of the area of the genetic FVIII mutation. In Chapter 8 the major findings of this thesis and potential implications are discussed and perspectives for future research to study the etiology of inhibitor formation are provided.

Samenvatting

Hemofilie A is een X-chromoom gebonden afwijking van het bloedstollingssysteem die wordt veroorzaakt door een tekort aan of afwezigheid van stollingsfactor VIII (FVIII). De bloedingsneiging die ontstaat in patienten met hemofilie A kan worden behandeld met intraveneuze toedieningen van FVIII. Een ernstige bijwerking die voorkomt in bij benadering 30% van de hemofilie A patienten is het herkennen van het toegediende FVIII als lichaamsvreemde stof door het immuunsysteem van de patiënt, hetgeen resulteert in de vorming van FVIII-remmende antistoffen. Deze antistoffen worden vaak "remmers" genoemd. Door de vorming van remmers is profylactische behandeling met FVIII niet langer effectief, als gevolg van de inactivatie van FVIII en de snellere klaring uit de bloedsomloop. Hoewel er verschillende genetische en behandelingsgerelateerde factoren zijn geïdentificeerd als risicofactoren voor remmervorming, is er weinig kennis beschikbaar over hoe en onder welke omstandigheden het afweersysteem van hemofilie A patienten het toegediende FVIII herkent en hoe dit in sommige patiënten kan leiden tot remmervorming en in andere patïenten kan leiden tot tolerantie voor FVIII.

Remmers zijn veelal antistoffen van het IgG isotype, met een hoge affiniteit voor FVIII. Voor de productie van dergelijke antistoffen is somatische hypermutatie en recombinatie van immunoglobuline genen door FVIII-specifieke B cellen vereist. Deze processen worden geïnitïeerd door de activatie van deze B cellen door FVIII-specifieke T cellen. De activatie van deze T cellen is op zijn beurt afhankelijk van de herkenning van FVIII-afgeleide peptiden gepresenteerd op MHC klasse II door antigeen-presenterende cellen (APC's), in combinatie met de herkenning van co-stimulatoire signalen afkomstig van de APC.

Het doel van dit proefschrift is om de mechanismen van FVIII endocytose en presentatie op MHC klasse II door antigeen-presenterende cellen te onderzoeken, met de bedoeling om nieuwe targets voor de modulatie van de remmervorming in hemofilie A patiënten te identificeren. In Hoofdstuk 1 wordt in algemene achtergrondinformatie voorzien met betrekking tot de vorming van remmers. In dit hoofdstuk wordt de huidige kennis over de risicofactoren voor remmervorming samengevat en worden de basisfuncties van een humorale immuunrespons weergegeven. De rol van APC's in de totstandkoming van een humorale respons tegen FVIII wordt verder toegelicht in Hoofdstuk 2. Dit hoofdstuk beschrijft de huidige kennis over de endocytose en de presentatie van FVIII op MHC klasse II door APC's.

In Hoofdstuk 3 wordt de bijdrage van verschillende receptoren aan de opame van FVIII door APC's onderzocht. Onze resultaten geven weer dat zowel de macrophage mannose receptor (MR), DC-SIGN en de Low Density Lipoprotein Receptor-Related Protein (LRP) in staat zijn om FVIII te binden, maar niet significant bijdragen aan de opname van FVIII door humane dendritische cellen. De opname van FVIII kon wel worden geremd door een monoclonale anti-FVIII antistof genaamd KM33, gericht tegen het C1 domein van FVIII. Een controle antistof gericht tegen het A2 domein van FVIII, genaamd VK34, was niet in staat om de opname van

FVIII te remmen. Deze resultaten suggereren dat KM33 een belangrijke regio in het C1 domein van FVIII afschermt die de opname door dendritische cellen medieert. Deze resultaten zijn bevestigd *in vivo*, gebuik makend van een hemofilie A muizenmodel. De aanwezigheid van antistof KM33 in de circulatie van deze muizen was in staat om deze muizen te beschermen tegen remmervorming na herhaalde toedieningen van FVIII, terwijl de aanwezigheid van antistof VK34 geen invloed had op de remmervorming.

Als vervolg op de in Hoofdstuk 3 gepresenteerde resultaten, zijn we op zoek gegaan naar de residuen in het C1 domein van FVIII die worden afgeschermd door KM33, om te bepalen of deze residuen de opname van FVIII door dendritische cellen mediëren. In Hoofdstuk 4 hebben we een FVIII variant gemaakt waarin C1 residuen 2090, 2092 en 2093 zijn vervangen door alanines. Deze variant, die een minimale binding aan KM33 vertoont, wordt significant minder opgenomen door muizen dendritische cellen dan wild-type FVIII. Herhaaldelijke toedieningen van deze FVIII variant aan hemofilie A muizen leidde tot een significant gereduceerde remmervorming dan toedieningen van wild-type FVIII.

De drie hieropvolgende hoofdstukken, hoofdstukken 5 tot en met 7, hebben betrekking op de gebeurtenissen die volgen op endocytose van FVIII. Deze hoofdstukken beschrijven de voorwaarden voor presentatie van FVIII peptiden op MHC klasse II en de herkenning van gepresenteerde FVIII peptiden door FVIII-specifieke CD4-positieve T cellen. In Hoofdstuk 5 introduceren we een nieuwe methode waarmee peptiden die gepresenteerd worden op MHC klasse II kunnen worden geïdentificeerddoor gebruik te maken van massa spectrometrie. Hier laten we zien dat humane dendritische cellen peptiden presenteren die afkomstig zijn van zowel endogene eiwitten als opgenomen eiwitten. De opame van FVIII leidt tot de presentatie van FVIII peptiden op een donor-afhankelijke manier. Sommige van deze peptiden worden gepresenteerd door meerdere donoren, terwijl andere peptiden alleen door een specifieke donor werden gepresenteerd. Deze donor-afhankelijke presentatie van FVIII peptiden kan worden verklaard door verschillen in HLA haplotype.

In Hoofdstuk 6 laten we zien dat sommige lokale factoren ook in staat zijn om de presentatie van FVIII peptiden te beïnvloeden. Dendritische cellen lijken beter in staat om FVIII peptiden te presenteren dan macrofagen, ondanks het feit dat beide celtypen gelijkwaardig zijn in de efficiëntie van FVIII opname. De aanwezigheid van antistoffen die in complex zijn met FVIII kan ook de efficiëntie van FVIII presentatie beïnvloeden. Complexvorming van FVIII met zware keten-gerichte antistof VK34 leidt tot een voorkeur voor de presentatie van lichte keten peptiden op MHC klasse II, terwijl complexvorming met lichte keten-gerichte antistof KM33 leidt tot de presentatie van uitsluitend zware keten peptiden. De aanwezigheid van verscheidene cytokines zoals IFN-γ, IL-6 en IL-10 tijdens de maturatie van dendritische cellen leidt ook tot veranderingen in het repertoire van gepresenteerde FVIII peptiden.

In Hoofdstuk 7 hebben we recombinante MHC klasse II tetrameren gebruikt om FVIII-specifieke CD4-positieve T cellen aan te tonen in twee niet gerelateerde milde hemofilie A patiënten met FVIII genotype Arg593Cys. Beide patiënten in deze studie hadden een DRB1*1101-specifieke T-cel respons gericht tegen een FVIII peptide bestaand uit residuen 589-608. Deze regio overlapt met de plaats van de genetische mutatie. Deze resultaten bevestigen eerdere bevindingen dat CD4-positieve T cellen in patiënten met milde hemofilie gericht zijn tegen een regio die de genetische mutatie omvat.
In Hoofdstuk 8 worden de belangrijkste bevindingen uit dit proefschrift en de potentiële implicaties besproken en worden suggesties gedaan met betrekking tot vervolgonderzoek naar de etiologie van FVIII-remmende antistoffen.

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Simon

Simon van Haren werd op 29 oktober 1981 geboren te Utrecht. In 2000 behaalde hij het VWO diploma aan College Blaucapel te Utrecht. In September van dat jaar is hij begonnen aan de studie Bio-medische wetenschappen aan de Universiteit van Amsterdam. In 2003 heeft hij zijn bachelor diploma behaald. Vervolgens is hij begonnen aan de master Bio-medical Sciences. Tijdens deze masteropleiding liep hij stage op de afdeling Medische Biochemie in het Academisch Medisch Centrum te Amsterdam, onder begeleiding van Dr. C.Williams en Dr. B.Distel. Tijdens deze stage deed hij onderzoek naar de rol van Pex5p bij peroxisomale eiwitimport in *S.Cerevisiae*. Daarna heeft hij stage gelopen in de School of Molecular Biosciences aan de University of Sydney, onder begeleiding van Dr. S. Easterbrook-Smith. Tijdens deze stage deed hij onderzoek naar de biochemische karakteristieken van het extracellulaire chaperone-eiwit Clusterin. Na het behalen van zijn doctoraal examen in 2006 is hij in datzelfde jaar begonnen aan zijn promotieonderzoek bij de afdeling Plasma Eiwitten van Sanquin Research te Amsterdam, onder begeleiding van Dr. J. Voorberg, Dr. A.B. Meijer en Prof.dr.K.Mertens. De resultaten van het promotieonderzoek zijn beschreven in dit proefschrift.

List of Publications

van Haren SD, Wroblewska A, Fischer K, Voorberg J, Herczenik E. Requirements for immune recognition and processing of factor VIII by antigen-presenting cells. Blood Reviews. 2011;In Press.

Herczenik E, van Haren SD, Wroblewska A, Kaijen P, van den Biggelaar M, Meijer AB, et al. Uptake of blood coagulation factor VIII by dendritic cells is mediated via its C1 domain. J Allergy Clin Immunol. 2011. In Press

van Haren SD, Herczenik E, ten Brinke A, Mertens K, Voorberg J, Meijer AB. HLA-DR-presented peptide repertoires derived from human monocyte-derived dendritic cells pulsed with blood coagulation factor VIII. Mol Cell Proteomics. 2011;10:M110 002246.

Williams CP, Schueller N, Thompson CA, van den Berg M, Van Haren SD, Erdmann R, et al. The Peroxisomal Targeting Signal 1 in sterol carrier protein 2 is autonomous and essential for receptor recognition. BMC Biochem. 2011;12:12.

James EA, van Haren SD, Ettinger RA, Fijnvandraat K, Liberman JA, Kwok WW, et al. T-cell responses in two unrelated hemophilia A inhibitor subjects include an epitope at the factor VIII R593C missense site. J Thromb Haemost. 2011;9:689-99.

van Haren SD, Voorberg J. Getting rid of bad memory. Blood. 2011;117:7-8.

van Helden PM, van Haren SD, Fijnvandraat K, van den Berg HM, Voorberg J. Factor VIII-specific B cell responses in haemophilia A patients with inhibitors. Haemophilia. 2010;16:35-43.

Diehl SA, Schmidlin H, Nagasawa M, van Haren SD, Kwakkenbos MJ, Yasuda E, et al. STAT3-mediated up-regulation of BLIMP1 Is coordinated with BCL6 down-regulation to control human plasma cell differentiation. J Immunol. 2008;180:4805-15.