Molecular determinants of FVIII immunogenicity in hemophilia A
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Modification of an exposed
loop in the C1 domain reduces
immune responses to factor VIII in
hemophilia A mice

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Abstract

Development of neutralizing antibodies to blood coagulation factor VIII (FVIII) provides a major complication in hemophilia care. In this study we explored whether modulation of the uptake of FVIII by antigen presenting cells can reduce its intrinsic immunogenicity. Endocytosis of FVIII by professional antigen presenting cells is significantly blocked by monoclonal antibody KM33, directed towards the C1 domain of FVIII. We created a C1 domain variant (FVIII-R2090A/K2092A/F2093A), which showed only minimal binding to KM33 and retained its activity as measured by chromogenic assay. FVIII-R2090A/K2092A/F2093A displayed a strongly reduced internalization by human monocyte-derived dendritic cells and macrophages, as well as murine bone marrow-derived dendritic cells. We subsequently investigated the ability of this variant to induce an immune response in FVIII-deficient mice. We show that mice treated with FVIII-R2090A/K2092A/F2093A have significantly lower anti-FVIII antibody titers and FVIII-specific CD4+ T cell responses when compared to mice treated with wild type FVIII. These data show that alanine substitutions at positions 2090, 2092 and 2093 reduce the immunogenicity of FVIII. Based on our findings we hypothesize that FVIII variants displaying a reduced uptake by antigen-presenting cells provide a novel therapeutic approach to reduce inhibitor development in hemophilia A.
Introduction

Over the past decades protein therapeutics such as hormones, enzymes, blood coagulation factors or antibodies have provided effective treatment for numerous diseases. Treatment commonly requires frequent high-dose administration of protein therapeutics and, although generally considered safe, they often induce immune responses. The factors that underlie immunogenicity of biomedical products can be related to the structure of protein, such as the presence of promiscuous T cell epitopes or post-translational modifications, but also to the formulation of the biomolecule. Treatment-related parameters such as dosage, frequency, route of administration as well as concomitant infections may also contribute to the induction of anti-drug immune responses. In patients with protein deficiencies administered therapeutics may be recognized by the immune system as non-self, thereby greatly increasing the risk of antibody development.

Hemophilia A is an X-linked bleeding disorder that is caused by a deficiency in blood coagulation factor VIII (FVIII). Conventional treatment comprising frequent administration of FVIII often results in formation of neutralizing antibodies, which inhibit FVIII activity. Both treatment-related factors, such as intensive treatment episodes, as well as genetic risk factors can contribute to the development of inhibitors. Polymorphic sites in genes involved in the adaptive immune response have been associated with anti-FVIII antibody formation. Development of high affinity IgG antibodies directed against FVIII is a CD4+ T cell-dependent process. Endocytosis of FVIII by professional antigen presenting cells (APCs) comprises the initial step leading to activation of helper T cells. Uptake and transfer of antigens through the lyso-endosomal pathway results in intracellular processing and presentation of FVIII-derived peptides on MHC II molecules to CD4+ T helper cells. Here we hypothesized that prevention of FVIII uptake by APCs will lead to diminished T and B cell responses. Previously we have shown that endocytosis of FVIII by APCs is mediated via its C1 domain, since administration of a monoclonal antibody directed towards an antigenic surface in the C1 domain reduced inhibitor titers in FVIII-deficient mice. Using an antibody-guided mutagenesis strategy we designed a C1 domain variant of FVIII which displayed a strongly reduced internalization by antigen presenting cells. In vivo studies revealed that this C1 domain variant showed decreased immunogenicity in a murine model for inhibitor development in hemophilia A. Our findings provide a novel paradigm for the reduction of the intrinsic immunogenicity of FVIII by modulating its uptake by antigen presenting cells.

Methods

Materials

Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden), CD14 microbeads (Miltenyi Biotech, Auburn, CA, USA) and human recombinant GM-CSF and IL-4 (both Cellgenix Technology Transfer, Freiburg, Germany) were used for generation.
of human monocyte-derived dendritic cells (MDDCs); M-CSF (PeproTech, Rocky Hill, NJ) was used to generate human monocyte-derived macrophages (MDMφ). For culturing murine bone marrow-derived dendritic cells (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); serum-free CellGro® DC medium was from CellGenix (Freiburg, Germany). Fetal calf serum was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Cell factories, culture flasks and 96-well microtiter plates were purchased from Nunc (Roskilde, Denmark). Ultrapure methanol-free paraformaldehyde was from Polysciences (Eppelheim, Germany). Antibodies used were: mouse IgG isotype control antibodies conjugated with FITC and PE (Dako, Glostrup, Denmark); mouse IgG isotype control IgG conjugated with APC, anti-human CD80-FITC, anti-human CD83-APC, anti-human CD86-APC, anti-human CD206-APC, anti-murine CD83-APC, anti-murine CD86, anti-murine CD11b-FITC, rat IgG isotype control antibody conjugated with FITC, APC or biotin, streptavidin-APC, anti-human CD16, anti-human CD32 and anti-human CD64 (BD Biosciences, San Jose, CA, USA); anti-human CD14-PE; anti-human IgG1-HRP (Sanquin Reagents, Amsterdam, The Netherlands); anti-human CD209-APC (AbD Serotec, Germany), anti-mouse CD14-biotin, anti-mouse CD45R-biotin, anti-mouse Gr-1-biotin and anti-mouse CD8 (eBioscience, San Diego, CA, USA); anti-human CD91-PE (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Anti-CD11c antibody producing cell line (clone HB-224) was purchased from ATCC (Manassas, VA, USA). Mouse monoclonal antibodies CLB-CAg117 and CLB-CAg9, and human recombinant antibodies EL14, KM33 and VK34 targeting different domains of FVIII have been described previously. For flow cytometry, antibody CLB-CAg117 was labeled with FITC using the FluoReporter® FITC Protein Labeling Kit (Invitrogen, Breda, The Netherlands).

Preparation of FVIII mutants
Construction of a B domain-deleted FVIII (del 746-1639) has been described previously. The remaining linker region (first five and last nine amino acids of the FVIII B domain) was identical for all variants prepared. Arg2090Ala, Lys2092Ala and Phe2093Ala substitutions were introduced by QuickChange site-directed mutagenesis. Coding regions of all constructs were verified by sequence analysis. HEK293 stable cell lines expressing recombinant proteins were prepared as described previously. All proteins were purified by immunoaffinity chromatography using anti-FVIII antibody VK34 coupled to CNBr Sepharose 4B as described previously. Protein concentration was measured by Bradford protein assay. FVIII concentration was determined by enzyme-linked immunosorbent assay essentially as described. FVIII activity was determined by a chromogenic assay according to manufacturer’s instructions (Chromogenix, Milan, Italy).

Binding of FVIII to KM33
FVIII binding to KM33 was measured by sandwich ELISA and using surface plasmon resonance analysis. For the ELISA assays, Nunc-Maxisorp 96-well plates were coated with KM33 IgG1 (5 μg/ml) antibody in coating buffer (50 mM
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NaHCO₃, pH 9.8) overnight at 4°C. Subsequently, FVIII variants were incubated in binding buffer (50 mM Tris pH 7.4, 1 M NaCl, 2% HSA) in a concentration range up to 1 U/ml on the plate. Bound FVIII was detected with HRP-conjugated mAb CLB-CAg117 (targeting C2 domain of FVIII) or CLB-CAg9 (targeting A2 domain of FVIII). Optical densities were measured at 450 nm with the subtraction of values obtained at 540 nm. Surface plasmon resonance analysis was performed using a BIAcore3000 biosensor system (GE Healthcare, Uppsala, Sweden) essentially as described. 22 Briefly, human monoclonal antibody EL-14 (27 fmol/mm²) was covalently coupled to the dextran surface of an activated CM5-sensor chip. Subsequently, FVIII variants (6.1 fmol/mm²) were loaded on EL-14 in 20 mM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid), 150 mM NaCl, 5 mM CaCl₂ and 0.005% Tween 20 (pH 7.4). Association and dissociation of 25 nM full-length antibody KM33 (IgG1) were performed in the same buffer at a flow rate of 20 μl/min for 4 minutes at 25°C. Association and dissociation curves were corrected for nonspecific binding to a channel coated with EL-14 only.

Hemophilic E17KO Mice
Hemophilic E17KO mice, characterized by a targeted disruption of exon 17 of the FVIII gene 23 were backcrossed into the C57BL/6J background as described previously. 24 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. 25

Generation of MDDCs, MDMΦ and BMDCs
Human MDDCs were prepared as described. 15 Blood of healthy individuals was drawn in accordance with Dutch regulations and following approval from Sanquin Ethical Advisory Board in accordance with the Declaration of Helsinki. For macrophage culture (MDMΦ), monocytes were resuspended at 2.5x10⁶ 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. Murine BMDCs were essentially prepared as described before. 26 Briefly, bone marrow cells were isolated by flushing femurs from E17KO mice with PBS supplemented with 2% FCS. After erythrocyte lysis, cells were resuspended at 1x10⁶ 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 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 mM glutamine and 10% FCS. Based on MHC class II and CD11c expression, over 95% of the cells generated by this method were DCs.

FVIII endocytosis by flow cytometry
FVIII endocytosis was measured as essentially described before using antibody CLB-CAg117 conjugated with FITC. 15 Saponin was used to permeabilize the cells and visualize internalized FVIII. FACS plots were analyzed using FlowJo software.
Administration of FVIII in hemophilic E17KO mice
Recombinant B-domain deleted human FVIII WT or mutants were diluted to 10 μg/ml in sterile PBS and a dose of 1 μg was administered intravenously in E17KO mice (n=8) five times weekly. Endotoxin levels were measured using a LAL chromogenic assay (ToxinSensor™, GenScript). 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
Levels of anti-FVIII antibodies in murine plasma were measured by ELISA and functional Bethesda assay essentially as described. 15 For the ELISA, 1 AU corresponds to signal obtained with 1 μg mouse monoclonal antibody CLB-CAg9. Data were analyzed using non-parametric Mann-Whitney U-test.

Analysis of anti-FVIII antibody-secreting cells by enzyme-linked immunosorbert spot assay (ELISpot)
Antibody secreting cells (ASCs) present in spleen were analyzed as described. 27,28

CD4+ T cell proliferation assay
Spleens collected after weekly injections of FVIII were processed into single-cell suspensions. Erythrocytes were removed and CD8+ cells were depleted by magnetic bead separation using beads coated with the anti-mouse CD8 antibody. 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 μg/ml streptomycin and 55 μM 2-mercaptoethanol in presence of increasing concentration of FVIII WT (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 [3H]thymidine for the last 18-20 hours. The results are expressed as the stimulation index defined as counts per minute (cpm) of cells incubated with FVIII divided by cpm of cells with medium only.

Statistical analysis
Unless stated otherwise, 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, ns – not significant.

Results
FVIII with alanine substitutions in positions 2090, 2092 and 2093 shows diminished binding to C1-domain directed monoclonal antibody KM33
We have previously shown that endocytosis of FVIII by human and murine APCs is blocked by human monoclonal anti-C1 domain antibody KM33 15 (see also supplemental Figure S1). Moreover, upon in vivo administration, KM33 was able to delay the immune response to FVIII. 15 These findings indicate that antibody
KM33 shields a binding site that is crucial for the uptake of FVIII by APCs. We explored which residues in the C1 domain are involved in binding of KM33 to FVIII. Lys2092 and Phe2093 have previously been implicated in KM33 binding. However, mutation of these residues to alanines does not completely abolish KM33 binding, therefore additional C1 domain amino acids might be involved in the interaction between FVIII and KM33. To test this hypothesis, a FVIII variant was prepared where, besides Lys2092 and Phe2093, we additionally substituted Arg2090 for an alanine (Figure 1). FVIII-R2090A, FVIII-K2092A/F2093A and FVIII-R2090A/K2092A/F2093A were expressed by stably transfected HEK293 cells and purified by affinity chromatography. Activities of all variants were similar to wild-type FVIII (FVIII WT), as measured by a chromogenic assay (Table 1).

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-K2092A/F2093A showed a significant reduction

<table>
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<th>FVIII</th>
<th>Specific activity, U/mg</th>
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<tr>
<td>Wild type</td>
<td>7.7 ± 1.9 x 10^3</td>
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<tr>
<td>FVIII-R2090A</td>
<td>6.2 ± 1.0 x 10^3</td>
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<tr>
<td>FVIII-K2092A/F2093A</td>
<td>8.3 ± 1.9 x 10^3</td>
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<tr>
<td>FVIII-R2090A/K2092A/F2093A</td>
<td>7.7 ± 0.8 x 10^3</td>
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Table 1. Specific activity of FVIII variants.
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C1 domain mutations alter FVIII endocytosis by antigen presenting cells

in ability to bind KM33 as compared to WT FVIII. Introduction of a single alanine substitution at position 2090 did not influence the binding, however the presence of this mutation additional to those in FVIII-K2092A/F2093A showed more pronounced decrease in binding to KM33 (Figure 2A and 2B). Moreover, we performed surface plasmon resonance analysis of the FVIII-KM33 interaction, where KM33 was passed over FVIII WT, FVIII-K2092A/F2093A and FVIII-R2090A/K2092A/F2093A. Similar to previous observations, FVIII-R2090A/K2092A/F2093A showed a greater reduction of binding to KM33 as compared to the variant with alanine substitutions at positions 2092 and 2093 (Figure 2C). Additionally, other control monoclonal antibodies directed towards various FVIII domains bound all three FVIII variants tested (supplemental Figure S4). Altogether, these results show that residues Arg2090, Lys2092 and Phe2093 are crucially involved in binding of KM33 to FVIII.

**Figure 2. 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 FVIII WT (●, solid line) or mutants: FVIII-R2090A (▲, dashed-dotted line), FVIII-K2092A/F2093A (■, dashed line) or FVIII-R2090A/K2092A/F2093A (◆, dotted line). Bound FVIII was detected either with C2-domain targeting antibody CLB-CAg117 (A) or A2-domain targeting antibody CLB-CAg9 (B). Data show mean ± SD of 3 experiments and are expressed as % maximum binding, where 100% corresponds to signal obtained with 1 U/ml FVIII WT. C. Surface plasmon resonance analysis of FVIII-KM33 interaction. KM33 IgG1 (25 nM) was passed over FVIII WT (solid line), FVIII-K2092A/F2093A (dashed line) or FVIII-R2090A/K2092A/F2093A (dotted line) immobilized on a sensor chip via C2 domain-targeting monoclonal antibody EL-14. Dissociation was initiated upon replacement of ligand solution by buffer. Data are representative of 3 independent experiments. WT – wild-type FVIII; 2090 – FVIII-R2090A; 2092/93 – FVIII-K2092A/F2093A; 2090/92/93 – FVIII-R2090A/K2092A/F2093A.
conjugated CLB-CAg117 antibody in the presence of saponin. Endocytosis of all tested FVIII variants by MDDCs or MDMΦ was significantly reduced as compared to FVIII WT (Figure 3A and 3B, white and grey bars, respectively). Interestingly, FVIII-R2090A/K2092A/F2093A showed decreased uptake when compared to FVIII-K2092A/F2093A, which indicates the critical importance of the additional mutation in position 2090. Similar findings were observed, when endocytosis was tested using murine bone marrow-derived dendritic cells (BMDCs) (Figure 3A and 3B, black bars). Moreover, when increased concentrations of variants were tested for endocytosis, uptake of FVIII-R2090A/K2092A/F2093A was significantly lower than that of the FVIII-K2092A/F2093A variant at all concentrations evaluated (Figure 3C). Altogether, these data indicate that Arg2090, Lys2092 and Phe2093 within the C1 domain are essential for the cellular uptake of FVIII by antigen presenting cells.

Figure 3. C1 domain mutations alter FVIII endocytosis by antigen presenting cells. A, B. Final concentration of 15 nM FVIII WT or mutants: FVIII-R2090A, FVIII-K2092A/F2093A or FVIII-R2090A/K2092A/F2093A was added to MDDCs (white bars), MDMΦ (grey bars) or BMDCs (black bars) for 30 minutes at 37°C. Internalized FVIII was detected by addition of CLB-CAg117-FITC in the presence of saponin. Representative histograms (grey filled histograms show untreated cells, open histograms represent cells treated with FVIII) (A) and quantification from at least 3 independent experiments (B) are shown (mean ± SD). Results are expressed as % MFI, where 100% corresponds to mean fluorescence intensity obtained with FVIII WT. C. Increasing concentrations (0-40 nM) FVIII WT (●, solid line), FVIII-K2092A/F2093A (■, dashed line) or FVIII-R2090A/K2092A/F2093A (◆, dotted line) were incubated with MDDCs at 37°C. 100% MFI refers to mean fluorescence intensity measured for 40 nM FVIII WT. Data show mean ± SD from 3 independent experiments. WT – wild-type FVIII; 2090 – FVIII-R2090A; 2092/93 – FVIII-K2092A/F2093A; 2090/92/93 – FVIII-R2090A/K2092A/F2093A.

Role of C1 domain in modulation of immune responses to FVIII in vivo

Residues 2090, 2092 and 2093 are important for cellular uptake of FVIII by both human as well as murine APCs. To investigate whether decreased endocytosis of FVIII-R2090A/K2092A/F2093A leads to reduced in vivo immune responses, FVIII⁻/⁻ mice (E17KO) were injected weekly with 1 µg of either FVIII WT or FVIII-R2090A/K2092A/F2093A. After 5 consecutive injections, the plasma of mice infused with FVIII WT contained high anti-FVIII antibody titers (ELISA: 252 ± 82 AU/ml, Bethesda: 364 ± 98 BU/ml), while the titer was significantly lower in the group that received
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Additional, to analyze the splenic anti-FVIII antibody secreting cells (ASCs), an ELISpot assay has been performed. In agreement with the antibody titers in the plasma, mice injected with FVIII WT had significant number of anti-FVIII ASCs, producing mainly IgG1 or IgG2b antibodies, whereas the group that received FVIII-R2090A/K2092A/F2093A had little or no FVIII-specific ASCs (Figure 5C). Moreover, administration of FVIII-R2090A/K2092A/F2093A led to reduced proliferation of splenic CD4^+ T cells upon in vitro restimulation with FVIII (7.2 ± 0.4 vs. 2.3 ± 0.3 SI for the highest FVIII concentration used, Figure 5D), while non-specific proliferation in presence of concanavalin A was similar for both groups (Figure 5E).

Figure 4. Reduction of immune responses upon in vivo administration of FVIII-R2090A/K2092A/F2093A in FVIII^−/− mice. Hemophilic E17KO mice (n=8) were injected intravenously 5 times weekly with 1 μg of FVIII WT or FVIII-R2090A/K2092A/F2093A. One week after last injections, mice were sacrificed and blood samples collected. A, B. Anti-FVIII antibody titers from the plasma samples were evaluated by ELISA (A) and Bethesda assay (B) as described in detail in "Methods". * P < 0.05 (non-parametric Mann-Whitney U test). C. The presence of splenic ASCs producing FVIII-specific antibodies was determined by ELISpot. As a control, IgG-producing ASCs were detected. Representative wells displaying both ASCs types, are shown for both groups. Spots were quantified using the AELvis reader and eli.analyse software. Group injected with FVIII WT – white bars, with FVIII-R2090A/K2092A/F2093A – black bars. D, E. CD8^− splenocytes were assayed in a thymidine (^3H) incorporation assay. Proliferation was measured after 72 hours and thymidine was added for the last 18-20 hours. Results are shown as stimulation index (SI) from triplicate wells (mean ± SD) for both FVIII-specific (D) as well as non-specific (E) proliferation. Mice injected with FVIII WT (●), group injected with FVIII-R2090A/K2092A/F2093A (○). WT – wild-type FVIII; 2090/92/93 – FVIII-R2090A/K2092A/F2093A.
Together, our results show that both B cell and CD4+ T cell responses are greatly reduced in mice that received FVIII-R2090A/K2092A/F2093A when compared to mice infused with wild type FVIII. Altogether, these findings clearly indicate that the immune recognition of FVIII by antigen presenting cells is a major determinant for its intrinsic immunogenicity. Therefore, FVIII variants displaying a reduced uptake by APCs could provide a novel therapeutic approach to reduce the risk of inhibitor development in patients with hemophilia A.

Discussion

Immune responses generated towards protein therapeutics represent a major complication in drug therapy. Production of high affinity antibodies to protein antigens requires priming of naive CD4+ T cells following recognition of peptide-sequences associated with MHC class II on antigen presenting cells. Several strategies such as selective modification of B and T cell epitopes aim for modulation of adaptive immune responses to biologicals. 3,29 Here, we sought to reduce immunogenicity by interfering with recognition and endocytosis of antigen by APCs. A number of studies have shown that enhancement of antigen endocytosis and/or subsequent presentation leads to stronger immune responses. 30-32 Therefore, we anticipated that reduction of antigen uptake will result in decreased immunogenicity of biotherapeutics. Indeed, we demonstrate that introduction of Ala substitutions in positions 2090, 2092 and 2093 results in a FVIII variant is poorly endocytosed by APCs and therefore yields lower anti-FVIII antibody titers in vivo. Decreased uptake of this FVIII-R2090A/K2092A/F2093A leads to reduced CD4+ T cell activation as well as diminished numbers of FVIII-specific antibody secreting cells. Together our findings suggest that prevention of endocytosis of FVIII by APCs comprises a powerful approach to reduce its immunogenicity.

Binding of FVIII to VWF protects FVIII from endocytosis by human DCs. 15,33 Therefore, changes in immunogenicity profile can be potentially explained by lack of or an increased binding of FVIII to VWF. However, binding of FVIII-R2090A/K2092A/F2093A to both human as well as murine VWF was similar to FVIII WT (supplemental Figure S2). In another report, the immunogenicity of FVIII was indirectly linked to its activity. 34 Thrombin, the end-product of the coagulation cascade, is capable of acting as a “danger” signal that is a necessary stimulus for APCs to activate CD4+ T cells. Given that FVIII-R2090A/K2092A/F2093A variant displays similar activity levels as FVIII WT as measured by a chromogenic assay, the reduction of immune response in mice infused with this variant is unlikely to be caused by a reduction in the amount of thrombin generated.

At present, it is not clear which endocytic receptors promote uptake of FVIII by antigen presenting cells. 15,35 Macrophage mannose receptor has been proposed as an important interaction partner of FVIII. 36 FVIII contains multiple potential glycosylation sites 37 with two major residues described to expose mannose-ending glycosylations located on the heavy (N239) and the light chain (N2118). 38,39 However, none of the FVIII variant tested in this study has modified
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glycans, yet their endocytosis is significantly impaired. This argues against mannose receptor being a major receptor for FVIII endocytosis by APCs. Broadly expressed scavenger receptor – low density lipoprotein (LDL) receptor-related protein (LRP) and other LDL receptor family members have been reported as important receptors for FVIII clearance. Lysine 2092 and phenylalanine 2093 have been shown to play a role in FVIII binding to LRP.20 FVIII-R2090A/K2092A/F2093A displays reduced affinity to LRP1 cluster II as compared to FVIII WT (supplemental Figure S3). Moreover, it has been suggested that disruption of FVIII-LRP binding could potentially lead to prolonged half-life of FVIII in vivo. However, despite the importance of these residues in endocytosis of FVIII by APCs, LRP has not been implicated in the uptake of FVIII by DCs. Moreover, we and others have previously shown that FVIII-K2092A/F2093A has reduced affinity to phospholipid membranes containing a low percentage of phosphatidylyl-serine (PS). However, since the PS-binding protein lactadherin does not compete for FVIII uptake by DCs, it is unlikely that this interaction plays a major role in the endocytosis of FVIII by APCs. All in all, despite the identification of a crucial determinant for endocytosis of FVIII, the precise mechanism by which FVIII is internalized by APCs remains to be established.

A number of other approaches have been used to induce tolerance or diminish inhibitor development in hemophilia A. Qian and co-workers demonstrated that disruption of the interaction between co-stimulatory molecules B7 and CD28 resulted in lower anti-FVIII antibodies titers. Also blockage of anti-CD40 has been shown to transiently suppress inhibitor development in murine hemophilia A. Other studies showed that using anti-CD3 antibody or co-injecting FVIII with anti-inflammatory drugs such as rapamycin can induce tolerance or reduce inhibitor development. Despite their promising outcome, these approaches are limited by their lack of antigen-specificity. Suppression of protective immune responses against incoming pathogens may present a potential side-effect of such treatment. B cell blasts expressing Ig-fusions of FVIII A2 and C2 domain have been successfully used to restore tolerance in hemophilic mice with pre-existing inhibitors. More recently, FVIII-expressing foamy virus transduced tolerogenic dendritic cells have been employed to reduce inhibitor titers in hemophilia A mice. However, concerns with respect to possible genotoxicity of viral vectors potentially limit the clinical application of these antigen-specific approaches to induce tolerance in hemophilia A.

Immune responses to intravenously administered FVIII are heterogeneous. Antibodies to FVIII develop in approximately 25% of patients with severe hemophilia who lack circulating endogenous levels of FVIII. The threshold for immune activation depends on FVIII genotype, but is also determined by the efficiency of immune recognition and processing of FVIII by antigen presenting cells and polymorphism in genes encoding proteins involved in adaptive immune responses. Our current findings document that the FVIII-R2090A/K2092A/F2093A variant is less efficiently endocytosed and displays lower immunogenicity as compared to wild type FVIII in mouse model of hemophilia A.
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Authorship Contributions
A.W. and S.D.H. designed the research, performed the experiments, analyzed the data, made the figures and wrote the paper. E.H. designed the research and analyzed the data. P.K. and A.R. performed the experiments. S.J. and X.L.Z. provided reagents. M.B., A.B. and A.B.M designed the research and analyzed the data. J.V. designed the research, analyzed the data and wrote the paper.

Disclosure of Conflicts of Interest
The authors declare no competing financial interests.
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References


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Supplemental material

Supplemental methods

Blocking experiments
To block the uptake of FVIII by human monocyte-derived macrophages (MDMΦ), prior incubation with cells (30 minutes, 37°C), 40 nM of monoclonal antibody VK34 or KM33 was first incubated with 10 nM FVIII for 30 minutes at 37°C.

Binding of FVIII to recombinant human and mouse VWF
Recombinant human VWF was purified as described before. Recombinant mouse VWF was prepared as reported previously. FVIII binding to VWF was measured by ELISA. Briefly, Nunc-Maxisorp 96-well plates were coated with 5 μg/ml either recombinant human or mouse VWF in 50 mM NaHCO₃ (pH 9.8) overnight at 4°C. Subsequently, FVIII variants were incubated on the plate in binding buffer (50 mM Tris pH 7.4, 150 mM NaCl, 5 mM CaCl₂, 2% HSA) in a concentration range up to 20 U/ml. Bound FVIII was detected with HRP-conjugated mAb CLB-CAg12 (targeting the A3-C1 domains of FVIII). Optical densities were measured at 450 nm with the subtraction of values obtained at 540 nm.

Surface plasmon resonance analysis
Surface plasmon resonance analysis was performed using a BIAcore3000 biosensor system (GE Healthcare, Uppsala, Sweden). For interaction studies of FVIII with VWF, mouse VWF was covalently coupled to the dextran surface of an activated CM5-sensor chip. Subsequently, various concentrations of FVIII variants (0.15-2 nM) were passed over immobilized VWF (10 fmol/mm²) at a flow rate of 20 μl/min. The interaction between FVIII and LRP was studied by using recombinant LRP1 cluster II. First, FVIII was immobilized onto a CM5 sensor chip precoated with FVIII C2 domain–targeting mAb EL14 (27 fmol/mm²). Then, various concentrations of LRP1 cluster II (6.25-200 nM) were passed over the immobilized FVIII with a flow rate of 20 μL/min. For both FVIII-VWF as well as FVIII-LRP interaction, nonlinear regression was used to fit the obtained data to a one-phase exponential association equation with GraphPad Prism 5.0 software (San Diego, Calif). The responses at equilibrium (Ymax) of each concentration of either FVIII (FVIII-VWF interaction) or LRP1 cluster II (FVIII-LRP binding) were then fitted by nonlinear regression using a one-site binding hyperbola to calculate apparent Kᵦ values. For binding of FVIII to various monoclonal antibodies, FVIII was immobilized onto CM5 sensor chip precoated with mAb EL14 (27 fmol/mm²). Association and dissociation of 100 nM full-length antibody KM33 (targeting C1 domain of FVIII), ESH4 (C2 domain), CLB-CAg12 (A3C1 domain) or CLB-CAg9 (A2 domain) were performed in the same buffer at a flow rate of 20 μl/min for 4 minutes at 25°C. Association and dissociation curves were corrected for nonspecific binding to a channel coated with EL-14 only.
**CD4⁺ T cell proliferation assay**

Spleens collected from E17KO mice after 5 times weekly i.v. injections of 1 μg FVIII were processed into single-cell suspensions. Erythrocytes were removed and CD8⁺ cells were depleted by magnetic bead separation using beads coated with anti-mouse CD8 antibody. Remaining CD8⁻ cells were cultured in round-bottomed 96-well plates for 48 hours in X-VIVO 15 medium supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin and 55 μM 2-mercaptoethanol in presence of FVIII (0.5 μg/ml), FVIII peptides (10 μg/ml) or concanavalin A (1 μg/ml). Proliferation was assayed by the addition of 1 μCi/well of [³H]thymidine for the last 18-20 hours. The results are expressed as counts per minute (cpm) of cells incubated with FVIII or peptides. FVIII peptides used in the assay: 2191-2210 (FVIII C2 domain), 2201-2220 (C2), 2211-2230 (C2), 2201-2230 (C2), 2076-2105 (C1), 2081-2100 (C1). FVIII C1 peptides contained either wild-type (WT) or mutated sequence (AAA: residues 2090, 2092 and 2093 were replaced by an Ala).

**Supplemental results**

**Endocytosis of FVIII by human monocyte-derived macrophages is mediated by the C1 domain**

Monoclonal antibody KM33 blocked the uptake of FVIII by MDMΦ whereas monoclonal antibody VK34 did not affect uptake of FVIII by MDMΦ (Figure S1). These results show that uptake of FVIII by MDMΦ is also mediated via its C1 domain.

![Figure S1](image)

**Figure S1.** Endocytosis of FVIII by MDMΦ is mediated by C1 domain. FVIII WT (10 nM) was preincubated either with C1-targeting antibody KM33 (grey bar) or A2-targeting VK34 (black bar) and subsequently added to MDMΦ. Internalized FVIII was detected using CLB-CAg117-FITC antibody in the presence of saponin. Results are expressed as % MFI, where 100% corresponds to mean fluorescence intensity obtained with FVIII alone (white bar). Mean ± SD are shown of three independent experiments. Representative histograms (grey filled histograms show untreated cells, open histograms represent cells treated with FVIII in presence or absence of antibodies) are shown on the right.

**Binding kinetics of FVIII variants to human and murine VWF and LRP**

To study the binding kinetics between murine VWF, LRP and FVIII, we performed surface plasmon resonance experiments, where either various concentrations of receptor fragment were passed over the sensor chip immobilized with FVIII or various FVIII concentrations were passed over immobilized VWF. Both FVIII WT and well as FVIII-R2090A/K2092A/F2093A were binding murine and human VWF in dose-dependent manner (Figure S2, A and B). Binding of FVIII WT and
C1 mutations alter response to FVIII in vivo

FVIII-R2090A/K2092A/F2093A to murine VWF was studied in more detail using surface plasmon resonance analysis. Different concentration of FVIII WT and FVIII-R2090A/K2092A/F2093A were passed over immobilized murine VWF (Figure S2, C and D, respectively). A dose dependent increase in binding of both FVIII WT and FVIII-R2090A/K2092A/F2093A was observed. The responses at equilibrium were used to calculate the binding parameters. Maximal binding was slightly lower for FVIII-R2090A/K2092A/F2093A when compared to FVIII WT. The apparent dissociation constant were below 1 nM for both WT FVIII and FVIII-R2090A/K2092A/F2093A (Figure S2, E and F), indicating that the binding affinity of FVIII-R2090A/K2092A/F2093A to mouse VWF is similar to that of WT FVIII.

We also determined the affinity of FVIII-R2090A/K2092A/F2093A for cluster II of LRP. We observed reduced binding for FVIII-R2090A/K2092A/F2093A to LRP1 cluster II when compared to WT FVIII (Figure S3). Calculated apparent $K_D$ value was significantly higher for FVIII-R2090A/K2092A/F2093A when compared to that of WT FVIII (Figure S3, D).
C1 mutations alter response to FVIII in vivo

Binding of a panel of antibodies to FVIII, FVIII-K2092A/F2093A and FVIII-R2090A/K2092A/F2093A

To exclude the possibility that observed differences in binding to KM33 is determined only by different binding of FVIII to EL-14, a panel of anti-FVIII monoclonal antibodies (KM33 – C1 domain, ESH4 – C2 domain, CLB-CAg12 – A3C1 domain, CLB-CAg9 – A2 domain) was passed over FVIII WT, FVIII-K2092A/F2093A and FVIII-R2090A/K2092A/F2093A immobilized on sensor chip via C2-targeting

**Figure S3. Binding of FVIII to LRP1 cluster II.** A, B. Various concentrations (6.25, 12.5, 25, 50, 100 and 200 nM) of LRP1 cluster II were passed over immobilized FVIII WT (A) or FVIII-R2090A/K2092A/F2093A (B). Dissociation was initiated upon replacement of ligand solution by buffer. C, D. The responses at equilibrium (Ymax) of each concentration of either FVIII WT or FVIII-R2090A/K2092A/F2093A (C) were used to calculate the apparent $K_D$ and $B_{MAX}$ values (D). Experiments were performed in triplicate.

**Figure S4. Surface plasmon resonance analysis of FVIII interaction with various anti-FVIII monoclonal antibodies.** Antibodies KM33, ESH4, CLB-CAg12 or CLB-CAg9 (100 nM) were passed over FVIII WT (solid line), FVIII-K2092A/F2093A (dashed line) or FVIII-R2090A/K2092A/F2093A (dotted line) immobilized on a sensor chip via C2 domain-targeting monoclonal antibody EL-14. Dissociation was initiated upon replacement of ligand solution by buffer. Data are representative of 3 independent experiments.
C1 mutations alter response to FVIII in vivo

Antibody EL-14. Only binding to KM33 was altered for FVIII mutants, while other antibodies showed similar binding to all three FVIII variants tested (Figure S4). These data show that FVIII-K2092A/F2093A and FVIII-R2090A/K2092A/F2093A react in a similar manner with EL-14, ESH4, CLB-CAg12 and CLB-CAg9. These findings also suggest that no major structural changes have occurred as a result of the substitution of residues 2090, 2092 and 2093 by an Ala.

Modification of residues Arg2090, Lys2092 and Phe2093 does not alter a major CD4+ T cell epitope

Reduced immune response observed for FVIII-R2090A/K2092A/F2093A in hemophilia mice could potentially result from elimination of a dominant T cell epitope. Therefore, we verified whether CD4+ T cells directed towards an epitope including these residues were present in hemophilia mice following multiple infusions with FVIII. CD8-depleted splenocytes isolated from immunized E17KO mice were stimulated either with FVIII C1 domain peptides 2076-2115 and 2081-2100 corresponding to either wild-type FVIII or FVIII-R2090A/K2092A/F2093A. In these latter peptides residues 2090, 2092 and 2093 were replaced by Ala. As a control we used peptides covering an established immunodominant T cell epitope in the C2 domain (region 2191-2230). FVIII and ConA were used as controls. In agreement with previous studies, proliferation in the presence of FVIII peptides was modest. Nevertheless, significant proliferation was observed only for C2 peptides (Figure S5). No specific proliferation was observed upon incubation of CD8-depleted splenocytes with peptides 2076-2105 and 2081-2100 (Figure S5). Also peptides 2076-2105 and 2081-2100 in which residues 2090, 2092 and 2093 were replaced by an Ala did not induce CD4+ T cell proliferation (Figure S5). These data suggest that region encompassing residues Arg2090, Lys2092 and Phe2093 does not comprise major CD4+ T cell epitope. Our findings indicate that the reduced immune response observed for FVIII-R2090A/K2092A/F2093A in hemophilia A mice is not caused by elimination of a dominant T cell epitope.

Figure S5. CD4+ T cells from immunized E17KO mice recognize FVIII C2 domain-derived peptides, but respond poorly to C1 peptides. CD8 splenocytes of hemophilia A mice injected with FVIII were assayed in a thymidine (3H) incorporation assay. Proliferation was measured after 48 hours and thymidine was added for the last 18-20 hours. Results are shown as counts per minute (cpm) from triplicate wells (mean ± SD). Ctrl indicates unstimulated cells. Black bars denote proliferation in response to peptides containing an established T cell epitope in the C2 domain. Grey bars represent proliferation in response to peptides overlapping residues Arg2090, Lys2092 and Phe2093. Proliferation in response to FVIII and ConA was included as a control.
Supplemental references


