Molecular determinants of FVIII immunogenicity in hemophilia A
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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 E17KO 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. Development of neutralizing antibodies (inhibitors) against FVIII occurs in approximately 20-40% of severe haemophilia A patients after replacement therapy, providing a major complication of haemophilia care. The antibody response is heterogeneous with antibodies binding to multiple domains of FVIII. 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. Subclass analysis revealed that anti-FVIII antibodies are composed of subclass IgG1 and IgG4. Clonal analysis revealed that the variable domains of anti-FVIII antibodies are extensively modified by somatic hypermutation. Both somatic hypermutation and isotype switching require the presence of antigen specific CD4+ T cells, which are enumerated following processing and presentation of FVIII derived peptides on MHC class II molecules on antigen presenting cells (APCs). Dendritic cells (DCs) are professional APCs that mediate uptake, intracellular processing and presentation of antigen to T cells. Processing of soluble antigens occurs by macrophages and resident DCs present in the spleen. Studies with human DCs have indicated that in vitro administration of FVIII does not result in activation and maturation of DCs. A slight increase in CD40 expression was noted upon administration of canine FVIII to bone marrow-derived murine DCs. Human DCs have been reported to internalize FVIII in a macrophage mannose receptor (MR, CD206) dependent manner. Mannose-ending glycans are present in the C1 (Asn2118) and A1 (Asn239) domains of FVIII and provide potential targets for carbohydrate recognition domains of C-type lectin receptors such as MR, DC-SIGN and dectin-2. Although a recent report suggests that LDL-receptor related protein (LRP) is not involved in the uptake of FVIII by dendritic cells, LRP is known as one of the main receptors engaged in clearance of FVIII. We previously described a panel of antibodies that were obtained from hemophilia patients. 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. 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 E17KO 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.
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, for details, see Online Repository Materials.

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 (5 µg/ml) was first immobilized on 96 well microtiter plates (Nunc, Roskilde, Denmark) overnight at 4°C in buffer containing 50 mM NaHCO₃, 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 CaCl₂, 2 mM MgCl₂ and 0.05% Tween-20, pH 7.4) and 5 µg/ml FVIII was added for 1 hour 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 F(ab’)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, 1 M 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 E17KO 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 E17KO 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 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. 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. Previously we have shown that these antibodies block the interaction of FVIII with LRP. 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. 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, SO₄-3-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. Altogether, our data indicate that LRP, MR and DC-SIGN are not essential for FVIII uptake.

Figure 3. Uptake of FVIII by MDDCs is independent of LRP, MR, and DC-SIGN. A. MDDCs were preincubated with α2m, RAP, mannan, or anti–DC-SIGN before 40 nM of FVIII was added to the cells. B. Receptor expression 72 hours after siRNA transfection was measured by using fluorescence-activated cell sorting. Grey histograms represent isotype controls, black lines indicate specific (LRP, MR and DC-SIGN) antibody stainings of cells with non-targeting (scramble) siRNA, dashed lines correspond to receptor staining after knockdown. C. 20 µg/ml of α2m-FITC, SO₄-3-Gal, or Btri used to monitor the endocytosis through targeted receptors. D. Internalized FVIII (0-80 nM of FVIII) was quantified by using fluorescence-activated cell sorting. 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 and the extracellular domain of MR. 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
(Figure 3 and see Figure E3). In addition, we tested the binding of FVIII to DC-SIGN-Fc (Figure 4). When increasing concentrations of LRP1 cluster II-Fc were added to immobilized 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. The KM33 F(ab’)2 fragment inhibited LRP1 cluster II-Fc binding to FVIII (Figure 4B). The addition of RAP efficiently blocked the interaction. As expected, α2m failed to compete with FVIII 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. 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 (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. When F(ab’)2 fragments of KM33 were added to FVIII, the interaction between MR CTLD4-Fc and FVIII

\[ \text{LRP1 cluster II-Fc} \]

\[ \text{MR Fc CTLD1-3-Fc} \]

\[ \text{MR Fc CTLD4-7-Fc} \]

\[ \text{DC-SIGN-Fc} \]

\[ \text{MR CR-FII-1-3-C} \]

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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). F(\(\text{ab}'\))\(_2\) fragments of KM33 at high concentrations (>5 \(\mu\)g/ml) significantly interfered with the interaction between DC-SIGN-Fc and FVIII (Figure 4F). Similar results were obtained when we replaced F(\(\text{ab}'\))\(_2\) 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

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**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.
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 E17KO 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. FVIII replacement therapy is no exception, since inhibitory antibody formation occurs in approximately 20% of the patients with severe haemophilia A. Both genetic risk factors and treatment-related parameters contribute to the etiology of inhibitor formation in haemophilia A. Induction of immune responses to FVIII subsequent to its intravenous infusion is most likely initiated in the spleen. 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. Subsequent uptake and processing of antigen results in presentation of peptides on MHC molecules allowing for instruction of naive T cells in the T cell zone of the white pulp. 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). 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. 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. 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 BMDCs observed in this and a previous study. Mannosylation of Asn239 and Asn2118 of FVIII has been implicated in the internalization of FVIII by DCs through binding to MR. 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 mannansensitive 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 E17KO 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.

**Key messages**
- An interactive surface in the C1 domain directs uptake of FVIII by dendritic cells.
- FVIII uptake by dendritic cells proceeds independently of LRP, DC-SIGN and MR.
- In vivo findings show that the interactive in the C1 domain is critical for initiation of immune responses to FVIII.

**Capsule summary**
Selective blockage of an interactive surface in the C1 domain of coagulation factor VIII prevents its uptake by dendritic cells and reduces immune responses in a murine model for hemophilia A.
Abbreviations

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<tr>
<td>FVIII</td>
<td>Blood coagulation factor VIII</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cells</td>
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<tr>
<td>MDDC</td>
<td>Human monocyte-derived dendritic cells</td>
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<td>BMDC</td>
<td>Murine bone marrow-derived dendritic cells</td>
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<tr>
<td>LRP</td>
<td>Low Density Lipoprotein receptor–related protein</td>
</tr>
<tr>
<td>RAP</td>
<td>Receptor-associated Protein</td>
</tr>
<tr>
<td>MR</td>
<td>Macrophage mannose receptor</td>
</tr>
<tr>
<td>SO₄⁻³-Gal</td>
<td>β-D-galactose-3-sulfate-PAA-fluor</td>
</tr>
<tr>
<td>Btri</td>
<td>Blood type B-PAA-fluor</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
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Online Repository Materials

Methods

Materials

Recombinant human FVIII was kindly provided by Dr. B.M. Reipert (Baxter Healthcare Corporation, Vienna, Austria). Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden), CD14 microbeads (MACS, Miltenyi Biotech, Auburn, CA, USA); human recombinant GM-CSF and IL-4 (Celligenix Technology Transfer, Freiburg, Germany) were used for generation of human monocyte-derived DCs (MDDCs). For culturing bone marrow-derived murine DCs (BMDCs), we purchased murine recombinant GM-CSF and murine recombinant IL-4 (R&D System, Minneapolis, MN, USA). The following reagents were used: FCS (HyClone, Thermo Fisher Scientific, Waltham, MA, USA), penicillin and streptomycin (Invitrogen), mouse IgG isotype control conjugated with FITC and PE (Dako, Glostrup, Denmark); mouse IgG isotype control 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 conjugated with FITC, APC or biotin, streptavidin-APC (BD Biosciences, San Jose, CA, USA); anti-human CD14-PE, human serum albumin (HSA, Cealb), anti-human IgG1-HRP (Sanquin Reagents, Amsterdam, The Netherlands); anti-human CD209-APC (AbD Serotec, Germany), anti-murine CD14-biotin, anti-murine CD45R-biotin and anti-murine Gr-1-biotin (eBioscience); ligand binding site blocking antibody against DC-SIGN (CD209), recombinant human DC-SIGN-Fc and LRP1 Cluster II-Fc chimeras (R&D System, Minneapolis, MN, USA); anti-CD91-PE (LRP, Santa Cruz Biotechnology, Santa Cruz, CA, USA); mannan, Lucifer yellow (Sigma-Aldrich, St. Louis, MO); methylamine-treated α2-macroglobulin (BioMac, Leipzig, Germany). CD11c producing cell line (clone HB-224) was purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). β-D-galactose-3-sulfate-PAA-fluor (SO$_4$-3-Gal) and Blood type B-PAA-fluor (Btri) were purchased from Lectinity (Moscow, Russia). Monoclonal antibodies CLB-CAg117, CLB-CAg9, CLB-CAg69 and CLB-CAg12 targeting different domains of FVIII have been described previously. Recombinant KM33, VK34, EL14 and control IgG1 (Cy1k) were cloned from ScFv fragments into full-length IgG1 molecules as described previously. Full-length antibodies were expressed in HEK-293 cells and purified using protein A Sepharose (GE Healthcare, United Kingdom). Bovine lactadherin was obtained from Haematologic Technologies, Vermont, USA. Recombinant VWF was purified from stably expressing HEK293 cell supernatant using monoclonal antibody CLB-RAg20. MR CTLD1-3-Fc and MR CTLD4-7-Fc was obtained as previously described.

FVIII uptake by flow cytometry

Approximately 2x10$^5$ of immature MDDCs or BMDCs were first washed once with serum-free medium and incubated with increasing concentrations of FVIII in 120 µl of serum-free IMDM medium for 30 minutes at 37°C. After FVIII uptake, cells
were washed once with ice-cold TBS, fixed with 1% freshly dissolved ultrapure methanol-free paraformaldehyde (Polysciences, Eppelheim, Germany) 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. To measure internalization capacity of MDDCs, 250 μg/ml Lucifer yellow was added to the MDDCs for 30 minutes. Mean fluorescence intensities and percentage of positive cells were determined by flow cytometry using LSRII (BD Biosciences, Uppsala, Sweden).

**Blocking experiments and siRNA**
To block uptake of FVIII, mannan, RAP, α2m, heparin and anti-DC-SIGN were first preincubated with MDDCs at 4°C for 30 minutes. Subsequently, 20 nM FVIII was added for 30 minutes at 37°C. For siRNA experiments, 4x10⁶ immature MDDCs were pulsed with 250 V, 150 µF and ∞Ω using 4 mm cuvettes in a Biorad GenePulser adding 6 μg non-targeting control- or LRP-, MR-, DC-SIGN-specific siRNA pools (Dharmacon, Thermo Fisher Scientific, Waltham, MA, USA) in serum-free medium on day 3. After 72 hours, the cells were analyzed for LRP, MR, DC-SIGN, CD80, CD83 and CD86 expressions. Knockdown of LRP, MR and DC-SIGN was confirmed by measuring endocytosis of FITC- or fluorescein conjugated selective ligands α2m-FITC, β-D-galactose-3-sulfate-PAA-fluor (SO₄₃-Gal) and Blood type B-PAA-fluor (Btrl), respectively. To study uptake, increasing concentrations (0-80 nM) of FVIII and 250 µg/ml Lucifer yellow (data not shown) were incubated for 30 minutes at 37°C with MDDCs. Mean fluorescence intensities were determined by flow cytometry.

**Surface Plasmon Resonance (SPR) analysis**
For SPR studies, we used recombinant LRP1 Cluster II. The interaction between FVIII and MR CTLD4-7-Fc or LRP1 Cluster II was studied using surface plasmon resonance (SPR) analysis with a BIAcore 3000™ biosensor (Biacore, AB, Uppsala, Sweden). First, FVIII was immobilized onto a CMS sensor chip precoated with FVIII C2 domain targeting monoclonal antibody EL14. Then, various concentrations of MR CTLD4-7-Fc (10-500 nM) or LRP1 cluster II (12.5-200 nM) were passed over the immobilized FVIII with a flow rate of 20 μl/minutes. We used non-linear regression to fit the obtained data to one-phase exponential association equation with GraphPad Prism 4.03 software (San Diego, CA, USA). The responses at equilibrium (Ymax) of each concentration of MR CTLD4-7-Fc or LRP1 cluster II were then fitted by non-linear regression using an one-site binding hyperbola to calculate Kᵦ values.

**Hemophilic E17KO Mice**
Hemophilic E17KO mice, characterized by a targeted disruption of exon 17 of the FVIII gene were backcrossed into the C57BL/6J background as described previously. Mice used in this study were male and aged between 6 and 25 weeks at the beginning of the experiments. The genotypes of hemophilic mice were confirmed by polymerase chain reaction analysis of genomic DNA extracted from ear clippings, as described previously.
Generation of human monocyte-derived (MDDC) and murine bone marrow-derived dendritic cells (BMDC)

Monocytes were isolated using CD14 microbeads and a magnetic cell separator from peripheral blood mononuclear cells of healthy donors with written consent isolated by Ficoll-Paque Plus. For siRNA experiments, where greater amounts of MDDCs were required, monocytes were isolated from peripheral blood mononuclear cells from aphaeresis samples using Elutra™ cell separation system as described previously. Monocytes (0.83x10^6 cells/ml) were differentiated into dendritic cells in IMDM medium supplemented with 1% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 1000 U/ml human recombinant GM-CSF and 800 U/ml human recombinant IL-4. After 4-6 days, immature phenotype of the cells was evaluated by determining cell surface markers CD14, CD80, CD83 and CD86. To obtain murine bone marrow-derived DCs (BMDC), bone marrow cells were isolated by flushing femurs from E17KO mice with PBS supplemented with 2% FCS. The bone marrow suspension was incubated in Tris-NH₄Cl at 4°C for 10 minutes to lyse erythrocytes. Finally, the cells were resuspended at 1x10^6 cells/ml containing 10 ng/ml murine recombinant GM-CSF and 0.3 ng/ml murine recombinant IL-4 and cultured for 7 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. Expression of CD11c, CD11b, CD80, CD86, CD14 and Gr-1 were routinely measured on day 7.

Quantification of internalized FVIII

To detect internalized FVIII light chain and heavy chain, MDDCs were lysed in ice-cold cells lysis buffer containing 150 mM NaCl, 2.5 mM CaCl₂, 0.1% Tween-20, 1% Nonidet P40, 2% HSA, 10 mM benzamidine, 5 mM N-ethylmaleimide and 50 mM Tris-HCl (pH 7.4). Cell lysates were centrifuged at 1000 g for 10 minutes. Lysates were diluted in lysis buffer lacking Nonidet P40 and incubated on ESH4 (monoclonal C2 domain antibody) or VK34 (monoclonal A2 domain antibody) coated microtiter plates for 1.5 hours at room temperature. Bound FVIII was detected using horseradish peroxidase conjugated CLB-CAg117 (C2) or CLB-CAg9 (A2).

Anti-FVIII inhibitory antibody measurements from mouse plasma by 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. Murine plasma dilutions were prepared in 50 mM Tris, 150 mM NaCl, 2% BSA, pH 7.4. Murine 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 by 1 µg of CLB-CAg9. To identify FVIII inhibitors, we performed Bethesda assay with Nijmegen modification. Data was analyzed using non-parametric Mann-Whitney U-test.
Quantification of KM33 in murine plasma
FVIII (5 μg/ml) was immobilized on 96 well microtiter plates in buffer containing 50 mM NaHCO₃, pH 9.8. Plates were blocked with 2% gelatin in PBS. Murine plasma dilutions were made in 50 mM Tris, 150 mM NaCl, 2% BSA, pH 7.4. Dilutions of purified KM33 IgG1 were used as a standard. Human anti-FVIII antibodies were detected with anti-human-IgG1-HRP (Sanquin reagents, Amsterdam, the Netherlands).

Online Repository Materials Results

FVIII endocytosis is blocked by VWF, phosphatidylserine does not influence FVIII uptake by immature MDDCs
To study the whether VWF is capable of interfering with FVIII endocytosis, we pre-incubated FVIII (20 nM) with 100 nM VWF for 15 minutes at 37°C prior to adding to MDDCs (Online repository material Figure 1). As expected, VWF blocked the uptake of FVIII. To investigate whether the annexin V-indistinguishable early phosphatidylserine patches play a role in the initiation of FVIII uptake, we added Lactadherin to the cells before (15 minutes at 37°C) uptake. We did not observe a significant effect.

Simultaneous knockdown of LRP, MR and DC-SIGN does not affect uptake of FVIII
To study the potential complementary role of LRP, MR and DC-SIGN in FVIII uptake, MDDCs were transfected with mixed siRNA pools targeting either LRP and MR or LRP, MR and DC-SIGN. Individual receptor expressions after 72 hours were similar to those after single knockdown (Figure 3B, data not shown), however, FVIII uptake was unchanged in case of both double (Online Repository Material Figure 2A) and triple (Online Repository Material Figure 2B) knockdown. Online Repository Material Figure 2C shows FVIII uptake after knockdown of individual receptors of their combinations expressed as percentages compared to 100% of 80 nM of FVIII endocytosed by cells transfected with non-targeting (scramble)
siRNA pools. These findings further confirm our observations with knockdown experiments for single receptors, i.e. that LRP, MR and DC-SIGN are not essential for FVIII endocytosis.

Online repository material Figure 2. Simultaneous knockdown of LRP, MR and DC-SIGN does not affect uptake of FVIII. A, B. Uptake of various concentrations of FVIII by MDDC transfected with non-targeting (scramble), LRP and MR (A), or LRP, MR and DC-SIGN (B) targeting siRNA pools. C. Uptake after knockdown is expressed as percentages of 80 nM FVIII endocytosed by cells transfected with non-targeting (scramble) siRNA pool. Data obtained from 2 or 3 individual experiments performed with cells from different donors.

Binding kinetics of LRP1 cluster II and MR CTLD4-7-Fc to FVIII
To study the binding kinetics between LRP, MR, DC-SIGN and FVIII, we performed surface plasmon resonance experiments, where various concentrations of receptor fragments were passed over the sensor chip immobilized with FVIII. We detected dose-dependent binding between FVIII and LRP1 cluster II (Online Repository Material Figure 3A) or MR CTLD4-7-Fc (Online Repository Material Figure 3B). The obtained apparent $K_D$ values were $5.15 \times 10^{-8}$ M and $2.50 \times 10^{-7}$ M, respectively (Online Repository Material Figure 3D), which were calculated from the amounts of bindings at equilibrium ($Y_{max}$, Online Repository Material Figure 3B and C). However, we need to acknowledge that the differences in affinity/avidity between a monomeric versus dimeric (Fc chimeric) analyte can be significant. $^{E17}$ Under the applied experimental conditions, we did not detect any interaction between immobilized FVIII and DC-SIGN-Fc (data not shown). These results illustrate that LRP1 cluster II and MR CTLD4-7-Fc both can interact with FVIII, however, the affinities of these interactions are relatively low, confirming their insignificant roles for uptake of FVIII.
Online repository material Figure 3. Binding of MR CTLD4-7-Fc and LRP1 Cluster II to immobilized FVIII. First, 12 nM FVIII was immobilized to the CM5 sensor chip through monoclonal antibody EL14. Then, LRP1 Cluster II (A) or MR CTLD4-7-Fc (B) were passed over the chip at 200 nM (1), 100 nM (2), 50 nM (3), 25 nM (4), 12.5 nM (5) concentrations of LRP1 Cluster II and 500 nM (1), 200 nM (2), 100 nM (3), 50 nM (4) and 10 nM (5) concentrations of MR-CTLD4-7-Fc. Ymax values obtained from one-phase exponential association alignments of LRP1 Cluster II (C) and MR CTLD4-7-Fc (D) binding curves were used to calculate the apparent $K_D$ and Bmax values (E). Experiments were performed in duplicates.
Online Repository Materials References


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