Molecular determinants of FVIII immunogenicity in hemophilia A  
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Immune complexes enhance uptake of blood coagulation factor VIII by antigen presenting cells

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Abstract

Development of antibodies directed against FVIII (referred to as FVIII inhibitors) is a common complication in hemophilia care. Immune tolerance induction (ITI) comprising frequent administration of high doses of FVIII is the treatment of choice for hemophilia A patients with inhibitors. Thus far, the molecular mechanisms contributing to tolerance induction have not been defined. Here we studied endocytosis of FVIII-containing immune complexes by antigen presenting cells. Bone marrow-derived dendritic cells (DC) were used as model antigen-presenting cells, as DCs are known for their ability to take up and process immune complexes via Fc gamma receptors (FcγRs). Bone marrow-derived murine DCs were able to efficiently take up FVIII pre-complexed with anti-FVIII antibodies (FVIII-IC) in a dose-dependent manner. Endocytosis of FVIII-IC was 3-6 fold more efficient when compared to equimolar concentrations of soluble FVIII. Moreover, enhanced endocytosis led to stronger FVIII-specific T cell proliferation. Analysis of subclass-specific anti-FVIII IgG revealed that IgG2a/ IgG2b antibodies were most potent in enhancing FVIII endocytosis. Uptake of FVIII-IC, but not FVIII alone, could be inhibited with 2.4G2 antibody indicating functional involvement of FcγRII/III in this process. These results were confirmed using murine DCs isolated from FcγR-deficient mice. Uptake of FVIII-IC was similar to that of FVIII in DCs derived from mice lacking all four FcγRs. Genetic ablation of FcγRII or FcγRIII did not affect the ability of anti-FVIII IgG to promote the uptake of FVIII. Collectively, these data provide further insight into the modulation of FVIII endocytosis by anti-FVIII antibodies.
Introduction

Hemophilia A is an X-linked bleeding disorder caused by a deficiency or dysfunction of blood coagulation factor VIII (FVIII). Patients with severe hemophilia A frequently suffer from spontaneous joint and muscle bleedings whereas patients with mild hemophilia A usually bleed only after trauma of surgery. \(^1\) Conventional treatment consists of frequent intravenous administration of FVIII. However, approximately 25% of patients with the severe form of hemophilia A develop an immune response resulting in the formation of antibodies that inhibit the procoagulant activity of FVIII. Anti-FVIII antibodies are primarily composed of IgG1 and IgG4 and have undergone affinity maturation through somatic hypermutation. \(^2\)-\(^5\) Both somatic hypermutation and class switching are dependent on CD4\(^+\) T cells which have been extensively characterized in both hemophilic mice \(^6\)-\(^10\) and hemophilia A patients. \(^11\)-\(^15\) Once inhibitor development occurs, infused FVIII can bind to circulating antibodies creating FVIII-specific immune complexes. Formation of immune complexes has been shown to modulate not only antigen half-life time in the circulation, but also immune responses in general. \(^16\)

Dendritic cells (DCs) are professional antigen-presenting cells specialized in induction of primary T cell response to foreign antigens in both MHC class I and II-dependent manner. \(^17\) DCs can capture antigens either in soluble form or through the uptake of larger particles such as exosomes, apoptotic or necrotic cells and antigen-IgG complexes. \(^18\) Typically, endogenous antigens are presented on MHC class I molecules for presentation to CD8\(^+\) T cells while peptides derived from exogenous, internalized antigens are loaded on MHC class II molecules and activate CD4\(^+\) T cells. Although macrophages and B cells also express MHC class II molecules and other proteins involved in MHC class II-dependent presentation, DCs have been exclusively shown to activate MHC class II-restricted CD4\(^+\) T cells in vivo after injection of immune complexes. \(^19\) Several studies have shown that IgG-complexed antigen is more efficiently cross-presented to MHC class I-restricted CD8\(^+\) T cells when compared to soluble antigens. \(^20\)-\(^23\)

Here, we studied whether FVIII-containing immune complexes (FVIII-IC) are efficiently endocytosed by dendritic cells. We show, that endocytosis of equimolar concentrations of FVIII-IC is much more efficient than FVIII alone. The enhanced uptake of FVIII-IC was not observed in DCs derived from Fc\(\gamma\)RI-IV deficient mice indicating that Fc\(\gamma\)R promote the uptake of FVIII-IC. Genetic ablation of neither Fc\(\gamma\)RII nor Fc\(\gamma\)RIII alone reduced the uptake of FVIII-IC suggesting that these receptors do not exclusively promote endocytosis of FVIII-IC.

Methods

Materials

For culturing murine bone marrow-derived dendritic cells (BMDCs), mouse recombinant GM-CSF (R&D System, Minneapolis, MN, USA) was used. Penicillin/streptomycin, RPMI-1640 and serum-free X-VIVO 15 medium were from Lonza
FVIII-immune complexes

(Walkersville, MD, USA. Fetal calf serum was purchased from Thermo Fisher Scientific (Waltham, MA, USA). 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: 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-mouse CD14-biotin, anti-mouse CD45R-biotin, anti-mouse Gr-1-biotin and anti-mouse CD8 (eBioscience, San Diego, CA, USA); monoclonal anti-FVIII antibodies ESH4 and ESH5 (Sekisui Diagnostics, Stamford, CT, USA). Anti-CD11c antibody producing cell line (clone HB-224) was purchased from ATCC (Manassas, VA, USA). Monoclonal antibodies CLB-CAgA, CLB-CAg9, CLB-CAg12 and CLB-CAg117 targeting different domains of FVIII have been described previously. 24-27

Fluorescent labeling of recombinant FVIII

Recombinant human FVIII was kindly provided by Dr. B.M. Reipert (Baxter Healthcare Corporation, Vienna, Austria). FVIII was labeled using the Microscale Alexa Fluor 488 protein-labeling kit (Invitrogen). Protein concentration and efficiency of labeling was spectrophotometrically determined at 280 and 495 nm. The degree of labeling was usually between 3 and 5 Alexa Fluor 488 molecules per 1 molecule of FVIII. The integrity of labeled FVIII (FVIII-488) was confirmed by SDS-PAGE (data not shown).

Mice

Hemophilic E17-KO mice, characterized by a targeted disruption of exon 17 of the FVIII gene 28 were backcrossed into the C57BL/6J background as described previously. 29 Genotype of hemophilic mice was confirmed by polymerase chain reaction analysis of genomic DNA extracted from ear clippings, as described previously. 6 FcγRIIb<sub>B6</sub>−/− 30, FcγRIII−/− 31, FcγRI/II/III/IV−/− and FcγRIIb<sub>B6</sub>flx/flx 30 mice were bred and maintained in the Animal Facility at Leiden University Medical Center. Unless stated otherwise, mice used in this study were male and aged between 6 and 12 weeks at the beginning of the experiment.

Generation of BMDCs

Murine BMDCs were essentially prepared as described before. 32 Briefly, bone marrow cells were isolated by flushing femurs with PBS supplemented with 2% FCS. After erythrocyte lysis, cells were resuspended at 1x10<sup>6</sup> 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.

Pulse loading of DCs with FVIII or FVIII–IgG complexes

FVIII–IgG immune complexes (FVIII-IC) were prepared by incubating recombinant human FVIII (Baxter) or recombinant human FVIII conjugated with Alexa Fluor 488 (FVIII-488) with a mixture of monoclonal anti-FVIII antibodies (CLB-CAgA,
CLB-CAg9, CLB-CAg12, CLB-CAg117, ESH4 and ESH5) for 30 min at 37 °C in a molar ratio 1:5. In uptake experiments, 0.2x10⁶ DCs were incubated with various concentrations of FVIII-488 or FVIII-488 pre-complexed with antibodies at 37°C for 1 hour in serum-free CellGro (CellGenix). Uptake was analyzed by flow cytometry (LSRII flow cytometer; BD Biosciences). Histograms were processed using FlowJo Version 7.6.5 software (TreeStar).

**Administration of FVIII in hemophilic E17-KO mice**
Recombinant human FVIII was diluted to 10 μg/ml in sterile PBS and 100 μl was administered intravenously in E17-KO mice.

**CD4⁺ T cell proliferation assay**
Spleens collected after 5 weekly injections of FVIII were processed into single-cell suspension. Erythrocytes were removed and CD8⁺ T cells, CD19⁺ B cells and CD138⁺ plasma cells were depleted by magnetic bead separation using anti-CD8, anti-CD19 and anti-CD139 beads. Remaining splenocytes were cultured in round-bottomed 96-well plates for 96 hours in X-VIVO 15 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 55 μM 2-mercaptoethanol in the presence of increasing concentration of FVIII or FVIII-IC to measure antigen-specific T cell 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 in medium supplemented 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**

**Endocytosis of FVIII immune complexes is more efficient than soluble FVIII**
Inhibitory antibodies develop in a subset of hemophilia A patients in response to replacement therapy. Circulating anti-FVIII antibodies readily bind to infused FVIII resulting in the formation of FVIII-specific immune complexes. It has been shown for the model antigen ovalbumin (OVA) that formation of immune complexes enhances endocytosis and leads to more efficient presentation and activation of specific T cells. In order to verify whether that phenomenon also holds true for FVIII, we evaluated internalization of FVIII and FVIII-IC in vitro using murine bone-marrow derived dendritic cells (BMDCs) as antigen-presenting cells. To enable efficient monitoring of endocytosis, recombinant FVIII was fluorescently labeled with Alexa Fluor 488. FVIII-specific immune complexes were formed using a panel of six mouse anti-FVIII monoclonal antibodies, directed against different domains of FVIII (Figure 1). A 5-fold molar excess of antibodies over FVIII was used to minimize the amount of non-complexed FVIII. As shown in Figure 2 (panel A and B), FVIII pre-complexed with antibodies (FVIII-IC) was efficiently
FVIII-immune complexes (FVIII-IC) were taken up by BMDCs in a dose-dependent manner. Moreover, endocytosis of FVIII-IC was much more efficient when compared to equimolar concentrations of soluble FVIII.

**FVIII-IC induce stronger FVIII-specific T cell proliferation when compared to non-complexed FVIII**

Subsequently, we studied whether enhanced endocytosis of FVIII-IC as compared to soluble FVIII will result in increased ability to prime FVIII-specific T cells, as it has been shown previously for ovalbumin-containing immune complexes. 19 FVIII-deficient mice were injected five times weekly with 1 µg of recombinant FVIII to enrich for FVIII-specific T cells. One week after last injection mice were sacrificed and isolated splenocytes were restimulated *in vitro* with increasing concentrations of either recombinant FVIII or FVIII pre-complexed with antibodies. Increased proliferation of FVIII-specific CD4+ T cells was observed upon incubation with FVIII-IC, as shown in Figure 3. Altogether, these data suggests that anti-FVIII IgG promotes endocytosis of FVIII by dendritic cells resulting in more efficient FVIII-specific T cell responses.

**Endocytosis of FVIII immune complexes proceeds via Fcγ receptors**

We subsequently investigated whether uptake of FVIII-IC is dependent on Fcγ receptors. BMDCs were first incubated with monoclonal antibody 2.4G2, blocking FcγR-mediated uptake by shielding a common epitope present in the extracellular domains of FcγRII and FcγRIII. 33 Next, increasing doses of FVIII-IC were added to the cells and incubated for 1 hour at 37°C. Flow cytometry analysis revealed that addition of 10 µg/ml 2.4G2 reduced the uptake of FVIII-IC by 80-90% for the highest concentration of FVIII used (Figure 4A). Uptake of FVIII
alone was not affected upon pre-incubation with the 2.4G2 antibody (Figure 4B and 4C). These findings show that Fcγ receptors mediate the uptake of FVIII when complexed with IgG.

To date, four different classes of FcγRs, known as FcγRI, FcγRIIb, FcγRIII and FcγRIV, have been identified in mice. 34 Three of them, namely FcγRI, FcγRIII and FcγRIV are activating receptors, whereas FcγRIIb is the only inhibitory one. To investigate the role of individual Fcγ receptors in endocytosis of FVIII-IC, we isolated bone marrow cells from various FcγR-deficient mice (Figure 5) and differentiate them into dendritic cells. FcγRIIb^{floxed/} mouse strain, expressing all functional Fcγ receptors, was used as a source of wild-type cells. Increasing concentrations of either FVIII or FVIII-IC were incubated with BMDCs for 1 hour

Figure 2. Endocytosis of FVIII immune complexes is more efficient when compared to non-complexed FVIII. A, B. Increasing concentrations (0-10 nM) of either soluble FVIII or FVIII pre-complexed with antibodies (FVIII-IC) were added to bone-marrow derived dendritic cells (BMDCs) and incubated at 37°C for 1 hour. Representative histograms are shown in A; gray-filled histograms show untreated cells, open histograms represent cells treated either with FVIII (upper panel) or FVIII-IC (lower panel). Results are expressed as % of mean fluorescent intensity (MFI) in panel B, where 100% refers to MFI measured for 10 nM FVIII-IC. Results from 3 independent experiments (mean ± SD) are shown.

Figure 3. FVIII-IC induce stronger FVIII-specific T cell proliferation when compared to soluble FVIII. Hemophilic E17-KO mice were injected intravenously 5 times weekly with 1 μg of recombinant FVIII. One week after the last injection mice were sacrificed, spleens were collected and processed into single-cell suspensions. Splenocytes were assayed in a thymidine (^{3}H) incorporation assay by in vitro restimulation with increasing concentrations of either FVIII or FVIII-IC. 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) and are representative for 2 independent experiments.
at 37°C and subsequently endocytosis was measured by flow cytometry. Single knock-out of either FcγRII or FcγRIII did not abolish the uptake of FVIII-IC (Figure 5A-C); however, in the absence of all four FcγRs, no increase in mean fluorescence intensity was observed upon addition of FVIII-IC to the cells, indicating functional involvement of Fcγ receptors in uptake of FVIII-IC (Figure 5D). Lack of the effect for single knock-out strains can potentially be explained by the redundancy of Fcγ receptors as reported previously. 19

Figure 4. Endocytosis of FVIII immune complexes depends on Fcγ receptors. A-C. Prior to addition of FVIII-IC, BMDCs were incubated with either 2.4G2 monoclonal antibody (10 µg/ml) or medium control for 15 minutes at 4°C. Subsequently, increasing concentrations of FVIII-IC (A) or a fixed concentration (5 nM) of FVIII or FVIII-IC (B and C) were added to the cells for 1 hour at 37°C. Representative histograms are shown in B: gray-filled histograms show untreated cells, open histograms represent cells treated either with FVIII (upper panel) or FVIII-IC (lower panel). Results are expressed as % of mean fluorescent intensity (MFI): in panel A 100% MFI refers to MFI measured for 10 nM FVIII-IC; in panel C 100% MFI refers to MFI measured for either FVIII or FVIII-IC alone (no 2.4G2 mAb added). Results are expressed as mean ± SD from 3 independent experiments.

Figure 5. Endocytosis of FVIII and FVIII-IC by BMDCs isolated from Fcγ-deficient mice. A-D. BMDC derived of FcγRIIb^floxflox^ (A), FcγRIIb^-/- (B), FcγRII^-/- (C) or FcγRI/II/III/IV^-/- (D) mice were used for these studies. Increasing concentrations (0-10 nM) of either soluble FVIII or FVIII pre-complexed with antibodies (FVIII-IC) were added to BMDCs and incubated at 37°C for 1 hour. Results are expressed as mean fluorescent intensity (MFI) and are representative of 2 independent experiments.
**IgG2a/2b antibodies are most efficient in promoting FVIII endocytosis**

The complexity in the FcγR family is mirrored by the presence of four different IgG subclasses (IgG1, IgG2a, IgG2b and IgG3), which bind with varying affinity and specificity to different FcγR receptors. \(^\text{35,36}\) FcγRI is the only high affinity receptor and binds mainly IgG2a; this isotype, together with IgG2b, is also bound by FcγRIII and IV, while IgG1 is exclusively recognized by FcγRIIb and FcγRIII. \(^\text{34}\) To unravel which IgG isotype predominantly mediates endocytosis of FVIII-IC by BMDCs, anti-FVIII antibodies were separated based on their isotype and incubated with recombinant FVIII as described previously. Subsequently, soluble FVIII or isotype-specific FVIII-IC were added to the cells for 1 hour at 37°C. IgG2a appeared to be more effective in enhancing endocytosis when compared to IgG1 (Figure 6). FVIII-IC comprised of a combination of IgG2a and IgG2b were most efficient in promoting FVIII-IC endocytosis (Figure 6), while a combination of IgG1 and IgG2b was less effective (Figure 6). Since IgG2a and IgG2b can bind to multiple FcγRs this also suggests that multiple FcγRs play a role in FVIII-IC endocytosis by BMDCs.

**Figure 6.** Anti-FVIII antibodies of subclass IgG2a and 2b are most efficient in promoting endocytosis of FVIII. Anti-FVIII monoclonal antibodies were divided based on their isotype (IgG1: ESH5 and CLB-CAg9; IgG2a: ESH4, CLB-CAgA and CLB-CAg117; IgG2b: CLB-CAg12) and subsequently incubated in molar ratio 1:5 with recombinant FVIII (2 nM) for 30 minutes at 37°C to allow formation of FVIII-immune complexes. Next, FVIII or FVIII-IC were added to BMDCs for 1 hour at 37°C. Results are expressed as % MFI, where 100% MFI corresponds to mean fluorescent intensity measured for FVIII alone. Results from 3 independent experiments (mean ± SD) are shown.

**Discussion**

Endocytosis of ICs by dendritic cells results in efficient processing and loading of antigen-derived peptides on MHC class I and/or II molecules and promotes subsequent induction of antigen-specific T cells. \(^\text{34}\) Immune tolerance induction (ITI) using high dosage infusion of FVIII is frequently used to eradicate inhibitory antibodies to FVIII in patients with hemophilia A. \(^\text{37}\) Infusion of FVIII in patients with anti-FVIII antibodies will result in rapid formation of FVIII-immune complexes (FVIII-IC). As yet our knowledge on the biological activity of FVIII-IC is limited. In this study we investigated the endocytosis of FVIII-IC by antigen-presenting cells as well as subsequent presentation and priming of FVIII-specific T-cells. We showed that FVIII-IC are more efficiently endocytosed when compared to uncomplexed FVIII. Subclass analysis suggested that anti-FVIII IgG2a and 2b promotes FVIII endocytosis more efficiently when compared to IgG1. Finally, enhanced endocytosis of FVIII-IC led to robust *in vitro* priming of FVIII-specific T cells. Altogether, these data suggest that formation of FVIII-IC reinforces already pre-existing anti-FVIII immune responses.
Endocytosis of FVIII-IC was FcγR-dependent as shown by blockage with monoclonal antibody 2.4G2, as well as employing bone marrow-derived DC derived from FcγRI-IV deficient mice. Genetic ablation of neither FcγRII nor FcγRIII alone affected the uptake of FVIII-IC, suggesting that these receptors do not exclusively promote FVIII-IC endocytosis. Both FcγRI and FcγRIII have been implicated in the enhanced uptake of ovalbumin-immune complexes. 

FcγRI binds with high affinity to monomeric IgG2a and therefore is constantly saturated with ligand, which would suggest that it might not play a major role in the uptake of immune complexes. Nevertheless, several recent experimental findings suggest that FcγRI is an important player in immunity. Simultaneous knockout of both FcγRI and FcγRIII, but not each of these separately, was shown to abolish MHC class I-restricted antigen presentation of ovalbumin-ICs, suggesting that the high-affinity FcγRI can cooperate with low-affinity FcγRIII in mediating endocytosis of immune complexes. The strong potentiating effect on FVIII uptake by IgG2a antibodies and the inhibitory effect of antibody 2.4G2 which neutralizes FcγRI via its Fc-tail is compatible with a possible role for FcγRI in the enhanced uptake of FVIII-IC. FcγRIV is also expressed on bone marrow derived DCs and binds with intermediate affinity to IgG2a and IgG2b. Therefore, we cannot fully exclude that FcγRIV also contributes to the uptake of FVIII-IC.

Extrapolating data obtained from mouse models into pathophysiological situations in patients is always challenging due to differences between mice and men. Based on the genomic localization and sequence similarity in the extracellular portion, mouse FcγRIII is most closely related to human FcγRIIa, while mouse FcγRIV seems to be the orthologue of human FcγRIIIa. Moreover, the affinity of the human FcγRs for the different IgG subclasses is significantly lower compared with their mouse counterparts. Anti-FVIII antibodies developing in hemophilia mice are mostly of IgG1, IgG2a and IgG2b isotype; IgG4 and IgG1 are the most abundant IgG subclasses in hemophilia patients with FVIII inhibitors. Titers of anti-FVIII IgG4 antibodies have been suggested to correlate with a poor prognosis of ITI therapy. IgG1 containing IC bind to all human FcγRs, while IgG4 containing IC bind primarily to human FcγRI, FcγRIIa/b/c and FcγRIIIa. However, formation of immune complexes by incubation of recombinant FVIII with panel of either mouse of human anti-FVIII monoclonal antibodies and subsequent addition to human monocyte-derived dendritic cells (moDCs) did not result in enhanced FVIII endocytosis, but led to inhibition of this process (data not shown). This could be potentially explained by limited expression of FcγRs on these cells (only FcγRIIa and IIa are present, see supplemental Figure S1A), whereas circulating conventional DCs, freshly isolated from peripheral blood also express high levels of activating FcγRI.

Monocyte-derived macrophages (MΦ) express a broader panel of FcγRs than moDCs, namely also FcγRI and FcγRII (supplemental Figure S1A). Consequently, when MΦ were used to study endocytosis of FVIII-IC, enhanced uptake was observed for FVIII-IC as compared to soluble FVIII (supplemental Figure S1B). Blocking antibodies showed major involvement of FcγRII, but also suggested that multiple FcγRs are needed for efficient endocytosis of FVIII-IC. Macrophages are
considered to mediate mostly clearance of ICs, while modulatory effects on antigen-specific immune response are thought to be strictly dependent on DCs. Therefore, additional studies employing human anti-FVIII antibodies and blood-derived conventional dendritic cells are needed to elucidate whether anti-FVIII IgG promotes the uptake of FVIII by human DCs. Nevertheless, despite the obvious difference between mice model systems and human, many of the basic principles and mechanisms underlying different IgG isotypes activities and role of FcγRs have been identified in mice and have been shown to hold true also in humans. Here, we showed that formation of immune complexes leads to more efficient delivery of FVIII to dendritic cells, and, as a consequence, more robust proliferation of FVIII-specific T cells. This data can provide further insight the modulation of immune responses to FVIII in hemophilia A.
References


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Supplemental data

Materials
Mouse monoclonal blocking antibodies anti-human CD16 (FcγRIII), CD32 (FcγRII) and CD64 (FcγRI) were from BD Biosciences (San Jose, CA, USA).

Generation of human monocyte-derived macrophages (MΦ)
For macrophage culture, monocytes isolated from blood of healthy donors 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. 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.

Blocking experiments
To block the uptake of FVIII, monoclonal antibody anti-CD16, anti-CD32 and/or anti-CD64 were first preincubated with MΦ at 4°C for 30 minutes. Subsequently, 20 nM of FVIII was added for 1 hour at 37°C. Cells were fixed and internalized FVIII was detected with monoclonal antibody CLB-CAg117-FITC in the presence of saponin. Mean fluorescence intensities were determined by flow cytometry.

Figure S1. Endocytosis of FVIII-IC by macrophages is dependent on FcγRI and FcγRII. A. Expression of Fcγ receptors on human monocyte-derived macrophages (upper panel) and DCs (lower panel). B. 5 nM of either soluble FVIII or FVIII-IC was incubated with MΦ for 1 hour at 37°C. C. Prior addition of FVIII-IC, cells were incubated with blocking antibodies directed to FcγRI, FcγRII and/or FcγRIII. Mean ± SD from 3 independent experiments are shown. Results are expressed as % MFI, where 100% corresponds to mean fluorescence intensity obtained with FVIII (A) or FVIII-IC (B).