

Factor VIII Fc Fusion Protein but not FVIII Drives Human Monocyte-Derived Dendritic Cell Activation via Fc γ R1a

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Abstract

This study compares the effect of recombinant Factor VIII Fc fusion protein (rFVIII-Fc) with recombinant FVIII (rFVIII) on monocyte-derived dendritic cells (moDC's). Cells treated with rFVIII-Fc showed morphological changes typical for cell activation, had a significant up-regulation of cell activation markers and produced higher levels of pro-inflammatory cytokines. Even after stimulation with Lipopolysaccharides, the addition of rFVIII-Fc led to increased expression of activation markers, indicating that rFVIII-Fc is capable of amplifying the maturation signal. On the contrary, cultivation of moDC's with rFVIII did not alter cell morphology or increase surface activation marker expression and pro-inflammatory cytokine production. The binding of the Fc domain to the activating Fc γ receptor 1a (Fc γ R1a) can cause cell activation. Therefore, the effect of rFVIII-Fc on Fc γ R1a was analyzed in detail. Cultivation of moDC's with rFVIII-Fc led to increased phosphorylation of Fc γ R1a, which was not detected for rFVIII. Blocking Fc γ R1a prior to the cultivation with rFVIII-Fc significantly reduced the activating effect of rFVIII-Fc, indicating that rFVIII-Fc-induced moDC activation was caused by Fc γ R1a. Moreover, rFVIII-Fc bound to FCGR2A-transfected human embryonic kidney 293 cells. Taken together, our data present a new mechanism of moDC activation by rFVIII-Fc via Fc γ R1a.

Introduction

Hemophilia A is an inherited bleeding disorder which, due to the lack of clotting FVIII, leads to frequent bleeding episodes. Until now, no cure for this disease exists but prophylactic treatment with plasma-derived or recombinant FVIII products has dramatically improved the life of hemophilia A patients. One of the biggest complications during treatment is the development of antibodies against the exogenous FVIII, which occurs in up to 30% of treated patients.¹ FVIII-neutralizing antibodies render the therapy ineffective and lead to increased bleeding episodes, higher morbidity and mortality in these patients.^{2,3}

Enormous efforts have been made in the recent years to develop new recombinant FVIII products which have an extended half-life, thereby reducing FVIII administration frequency and

improving patients quality of life.⁴ However, any modification to the FVIII sequence poses the risk of causing unwanted immunogenicity. Thus, extensive *in silico*, *in vitro*, and *in vivo* analyses are required to evaluate the immunogenicity of each of these new products before they go into clinics.

The recombinant B-domain-deleted FVIII product Efmoroctocog alfa (marketed as Elocta[®] and Elocate[®]) contains a dimeric constant Fc region of human IgG1. This Fc fusion has been reported to prolong the therapeutic half-life through binding to the neonatal Fc receptor (FcRn).^{5,6} Although the way the Fc domain prolongs FVIII's half-life is known, its effect on immunogenicity is still a matter of debate. While tolerogenic properties of the Fc portion of IgG have been described over the past decades,⁷ it is well documented that various therapeutic monoclonal antibodies induced neutralizing antibodies.⁸ Moreover, Fc fusion constructs have been utilized in vaccine development strategies to enhance the immune response towards the immunizing antigen.^{9–11} Neo-epitopes generated at the hinge region, modified uptake and processing as well as a different bio-distribution of the fusion protein could create a setting which triggers an unwanted immune response.¹²

The Fc domain not only interacts with the half-life-increasing FcRn, but can also engage in an interaction with various members of the Fc γ receptor (Fc γ R) family. These receptors are expressed on different immune cells and bind the Fc domain of IgG. In humans, the Fc γ R family is composed of four activating receptors (Fc γ RI, Fc γ R1a, Fc γ R1c and Fc γ R1a) and one inhibitory receptor (Fc γ RIIb), triggering or inhibiting immune functions, respectively.¹³ Fc γ RI is the only Fc γ R which binds monomeric IgG with high affinity. The other Fc γ Rs are low-affinity IgG receptors, which interact with antibodies present as immune complexes.¹⁴

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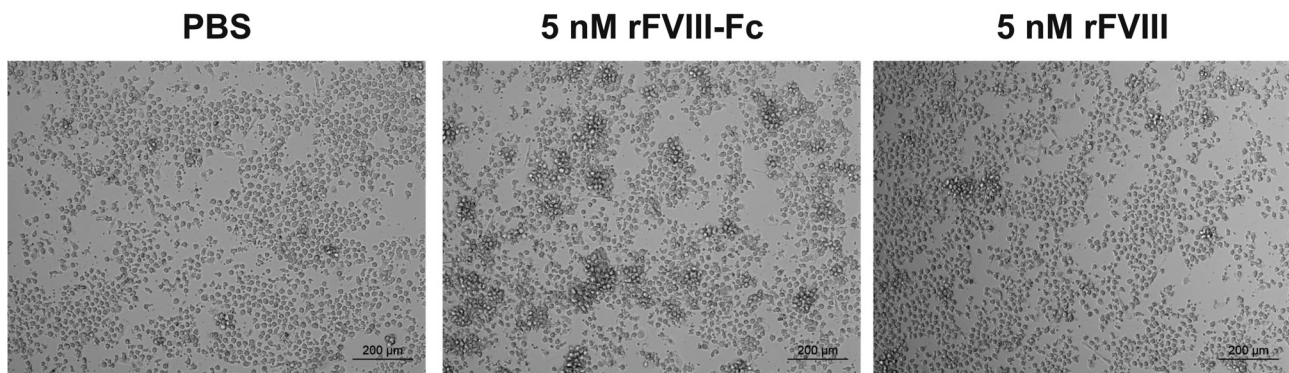


Figure 1. Morphological changes induced in moDC's upon treatment with rFVIII-Fc and rFVIII. moDC's were cultivated with 5 nM rFVIII-Fc, 5 nM rFVIII or PBS. Images were taken 23 hours post treatment with the Axio Observer Z1 microscope using a 100-fold magnification. Displayed are images from one representative donor.

Dendritic cells (DC's), which are the most efficient antigen-presenting cells, co-express the activating receptor FcγRIIa as well as the inhibitory receptor FcγRIIb. The balance between these 2 opposing receptors determines whether DC's are activated, mature and provide signals to activate T cells and subsequently trigger an immune response.¹⁵ Recombinant FVIII (rFVIII) itself failed to induce cell maturation in the in vitro model of monocyte-derived DC's (moDC's).¹⁶ However, since the Fc domain of rFVIII-Fc is of the IgG1 subclass, it is able to interact with FcγRIIa and FcγRIIb, possibly resulting in an activating or inhibitory signal for DC's. Thus, this study aimed to compare the effect of rFVIII-Fc and rFVIII on moDC maturation by analyzing morphological signs of cell activation, measuring the expression of co-stimulatory molecules as well as the production of pro-inflammatory cytokines. Furthermore, binding studies of rFVIII-Fc and rFVIII with FCGR2A-transfected human embryonic kidney (HEK) 293 cells were carried out. The involvement of FcγRIIa in moDC activation was assessed by measuring the receptor phosphorylation and performing inhibition experiments with an FcγRIIa-specific blocking antibody.

Results

rFVIII-Fc but not rFVIII induces moDC activation and pro-inflammatory cytokine production

DC's are the sentinels of the immune system, constantly monitoring their environment for foreign structures such as pathogens or other non-self proteins. Upon recognition and uptake of foreign structures, immature DC's are converted into mature, potent antigen-presenting cells. This maturation process is accompanied by morphological changes, such as cell clustering, the up-regulation of the expression of molecules involved in antigen presentation (HLA-DR) and co-stimulatory molecules (CD40, CD80, CD86), in addition to the production of various cytokines.

It has been reported that recombinant naïve FVIII itself does not induce DC activation and maturation in vitro.¹⁶ In order to see if this is also true for the FVIII-Fc fusion protein, moDC's generated from healthy donors were incubated for 23 hours with 5 nM rFVIII-Fc, 5 nM rFVIII or PBS and analyzed by light microscopy for morphological alterations related to DC activation and maturation. Interestingly, substantially more cell clustering was observed following rFVIII-Fc treatment compared

to rFVIII or PBS treatment (Fig. 1). Clusters of DC's have been observed following treatment with various maturation inducing agents,^{17–19} indicating that rFVIII-Fc induces cell activation.

In order to confirm if the observed morphological changes following rFVIII-Fc treatment are also reflected by the expression of relevant cellular activation markers, the level of CCR7, CD40, CD80, CD86, CD274, and HLA-DR were determined by flow cytometry. Cells treated with PBS served as a control and were used for normalization. As shown in Figure 2, surface expression of the maturation markers HLA-DR, CD40, CD80, and CD86 increased with rFVIII-Fc concentrations above 0.5 nM for most donors, with CD40 and CD80 being significantly upregulated compared to PBS-treated cells. On the contrary, no significant alteration in the expression of these maturation markers was observed for moDC's treated with rFVIII. The expression of CCR7, a C-C chemokine receptor usually up-regulated upon DC maturation declined and the expression of the inhibitory receptor ligand CD274 significantly increased upon rFVIII-Fc treatment. rFVIII had no effect on the expression of CCR7 and CD274. The overall viability of the cells treated with the different concentrations of rFVIII-Fc and rFVIII was not altered (SDC, Fig. 1, <http://links.lww.com/HS/A58>).

DC activation and maturation is usually accompanied by the production of cytokines. Therefore, the amount of the pro-inflammatory cytokines IL-6 and IL-8 was analyzed in the supernatant of rFVIII-Fc- or rFVIII-treated moDC's 23 hours post stimulation via the cytometric bead array. As shown in Figure 3, the levels of both cytokines were strongly increased when the cells were treated with rFVIII-Fc. At 5, 10, and 20 nM rFVIII-Fc a statistically significant difference between rFVIII-Fc-treated cells and PBS-treated cells was observed for IL-6. Interestingly, for 3 out of 5 donors very high levels of IL-8 were detected upon rFVIII-Fc treatment. Cells treated with rFVIII showed no statistically significant difference in IL-6 and IL-8 levels compared to PBS-treated cells. On average, the IL-6 and IL-8 levels for rFVIII-Fc-treated cells were more than 10 times higher than for rFVIII-treated cells. Taken together, the increased secretion of pro-inflammatory cytokines following rFVIII-Fc treatment is in line with the previous finding that moDC's are activated by rFVIII-Fc.

Since foreign proteins exert their immunogenicity only within a certain micro-environment, lipopolysaccharide (LPS) from *Pseudomonas aeruginosa* was added to the moDC's following a 3-hour incubation period with rFVIII-Fc or rFVIII. As shown in

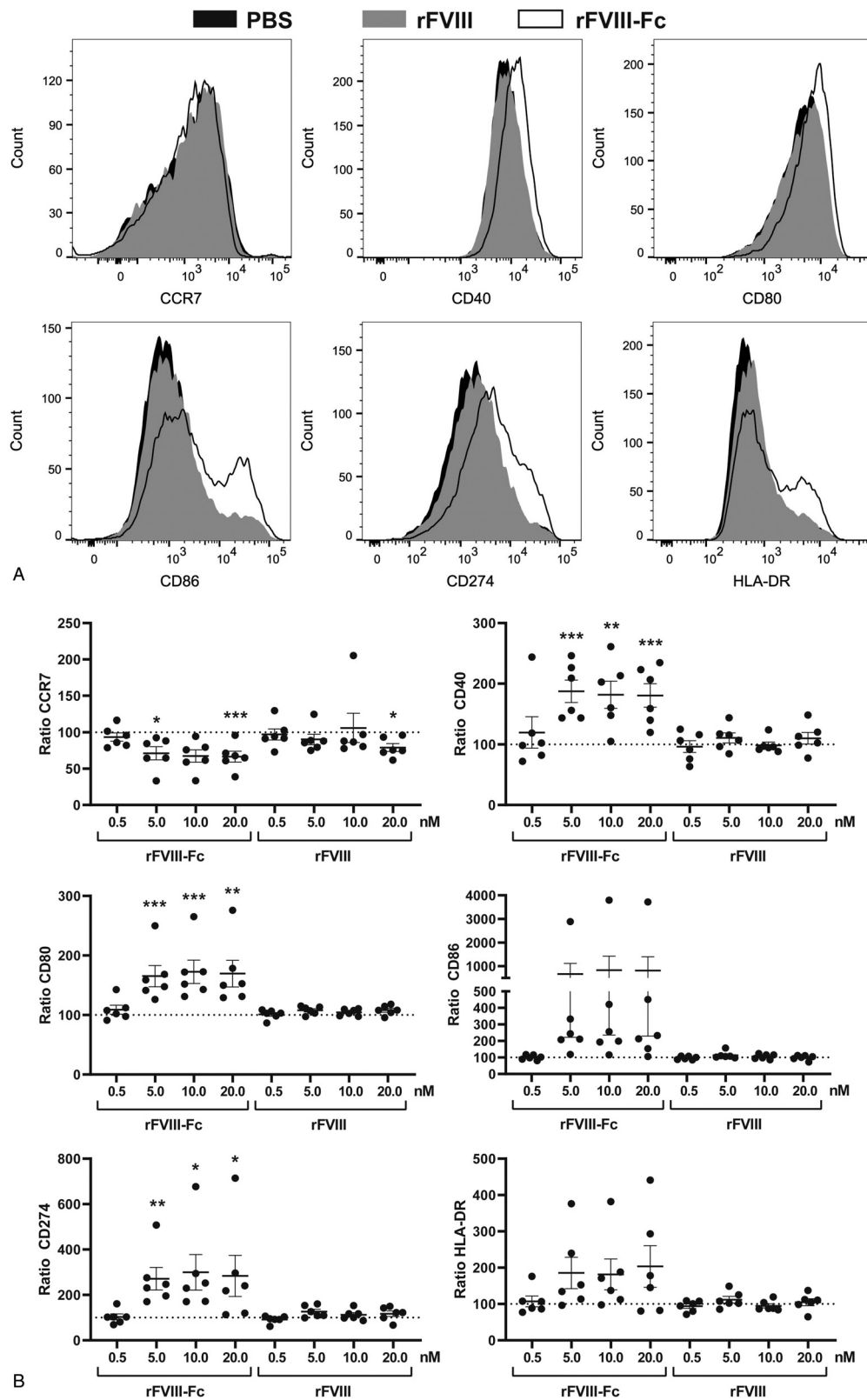


Figure 2. Effect of rFVIII-Fc and rFVIII on the expression of activation markers on moDC's. moDC's were cultivated with 0.5, 5, 10, and 20nM rFVIII-Fc or rFVIII. PBS-treated cells served as a control. Expression of CCR7, CD40, CD80, CD86, CD274, and HLA-DR was determined by flow cytometry on viable, single cells. (A) Displayed are representative histograms of one donor treated with 10nM rFVIII-Fc (black line, no filling), rFVIII (grey filled) or PBS (black filled). (B) Summary of the changes in surface expression of moDC obtained from 6 healthy donors treated with rFVIII-Fc or rFVIII. Data are presented as mean percentage of change in MFI of FVIII-stimulated cells over MFI of PBS-stimulated cells \pm SEM with each dot representing one donor. Data of each concentration were analyzed using one-way ANOVA followed by Dunnett Multiple Comparison test relative to cells treated with PBS (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

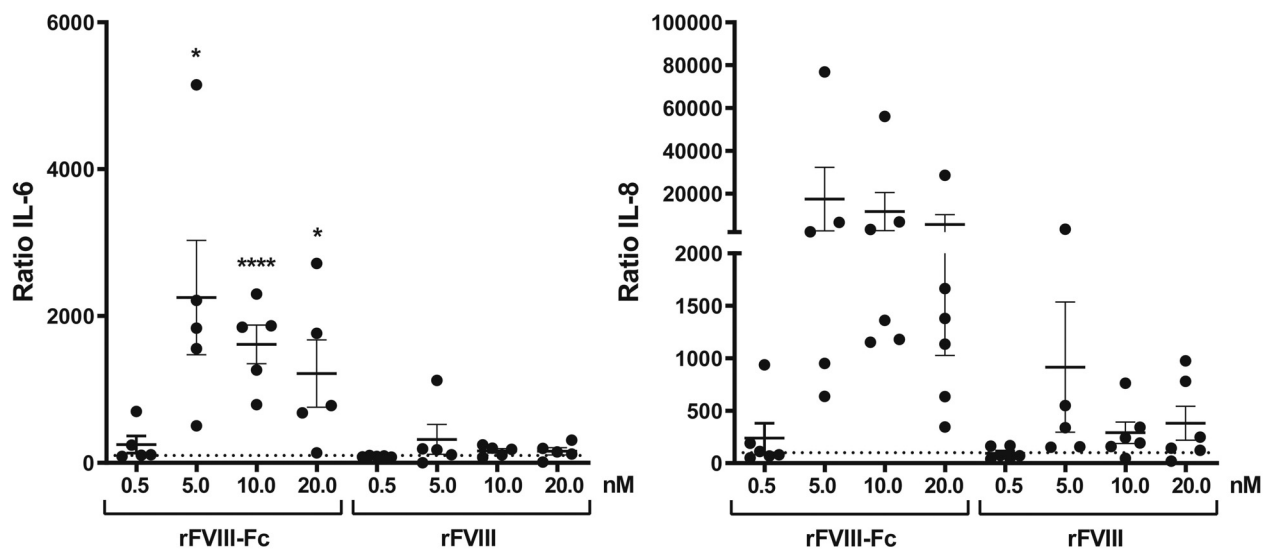


Figure 3. Effect of rFVIII-Fc and rFVIII on IL-6 and IL-8 levels. moDC's were cultivated with 0.5, 5, 10, and 20 nM rFVIII-Fc or rFVIII for 23 hours. PBS-treated cells served as a control. IL-6 and IL-8 concentrations were determined simultaneously via cytometric bead array. Data are presented as mean percentage of change in cytokine amount of FVIII-stimulated cells over cytokine amount of PBS-stimulated cells \pm SEM with each dot representing one donor. To evaluate if a difference between PBS-, rFVIII-Fc- or rFVIII-treated cells in the cytokine level exists, data of each concentration were analyzed using one-way ANOVA followed by Dunnett Multiple Comparison test relative to cells treated with PBS (* $p \leq 0.05$, **** $p \leq 0.0001$).

Figure 4, rFVIII-Fc led to a small but significant additional increase in the expression of the maturation markers CD80, CD86, and CD274 when the cells were stimulated with LPS. In conclusion, the LPS-induced maturation signal was amplified by rFVIII-Fc, but not rFVIII.

rFVIII-Fc fusion construct is required for moDC activation

To determine whether a combination of non-covalently bound Fc- and rFVIII-proteins induce a similar moDC activation as seen for rFVIII-Fc, mixtures of 5 or 10 nM human IgG1 Fc and 5 nM rFVIII were added simultaneously to the cells (Fig. 5). Analysis of different activation markers showed no statistically significant difference between cells incubated with rFVIII in the presence or absence of IgG1 Fc. Compared to rFVIII alone, a slight tendency towards increased IL-6 and IL-8 levels was detected, when rFVIII was applied together with 5 nM IgG1 Fc to the cells. However, the increase in IL-6 and IL-8 was not statistically significant and much lower compared to rFVIII-Fc-treated cells. No effect was observed when rFVIII was added together with 10 nM IgG1 Fc. As seen before, incubation of the cells with rFVIII-Fc showed a significantly higher expression of CD40, CD80, CD86, CD274, and HLA-DR and higher levels of IL-6 and IL-8 compared to cells treated with rFVIII. These findings indicate that the strong and potent activation of the moDC's is induced by the covalently-linked FVIII-Fc fusion construct, but not by similar concentrations of a mixture of IgG1 Fc and rFVIII.

Fc fusion proteins can bind to both activating and inhibitory FcγR. Since Fc engagement with activating receptors of the FcγR family has been reported to result in immune cell activation, it is likely that the observed activation of moDC's by rFVIII-Fc is mediated by an interaction with an activating FcγR. The presence of the different FcγR on moDC's was assessed by flow cytometry and is shown in Figure 5C. While expression of FcγRI and FcγRIII was absent or low, moderate to high levels of FcγRII

(FcγRIIa and FcγRIIb) were detected on moDC's. Thus, FcγRII is a potential candidate for triggering cell activation following rFVIII-Fc engagement. Moreover, a notably stronger activation following rFVIII-Fc treatment observed in 2 donors (highlighted in Fig. 5 in dark and light red) correlated with higher expression levels of FcγRII. The expression of the macrophage mannose receptor (CD206), which has been implicated in FVIII uptake,^{20,21} was not increased in these 2 donors. Taken together, these findings support the hypothesis that FcγRII is involved in the rFVIII-Fc-mediated activation of moDC's.

rFVIII-Fc binds to FcγRII on FCGR2A-expressing HEK293 cells but binding to moDC's does not depend on FcγRII

FVIII binding to moDC's has been reported to be mediated by various cell surface receptors, such as the low density lipoprotein receptor-related protein (LRP1), CD206, Siglec-5, galectin-1 and galectin-3.²² Since moDC's express the low-affinity FcγRII, differences in binding between rFVIII-Fc and rFVIII could occur. In order to compare the binding of rFVIII-Fc and rFVIII, moDC's were incubated with increasing FVIII concentrations at 4°C. The binding of rFVIII-Fc and rFVIII was detected with a biotin-labeled FVIII-specific nanobody, followed by an incubation with a APC-streptavidin. A concentration-dependent binding of rFVIII-Fc to moDC's was observed, although 100 nM of rFVIII-Fc were not sufficient to reach saturation (Fig. 6 (A)). Interestingly, the binding characteristics of rFVIII appeared to be similar to those of rFVIII-Fc.

Competition experiments in which the binding of 10 nM rFVIII-Fc was inhibited by the addition of increasing concentrations of rFVIII are shown in Figure 6 (B). rFVIII-Fc binding was detected with an Fc-specific antibody. rFVIII-Fc binding was efficiently inhibited with increasing concentrations of rFVIII. A decrease in rFVIII-Fc binding was detected at concentrations of more than 10 nM. Taken together, these experiments indicate

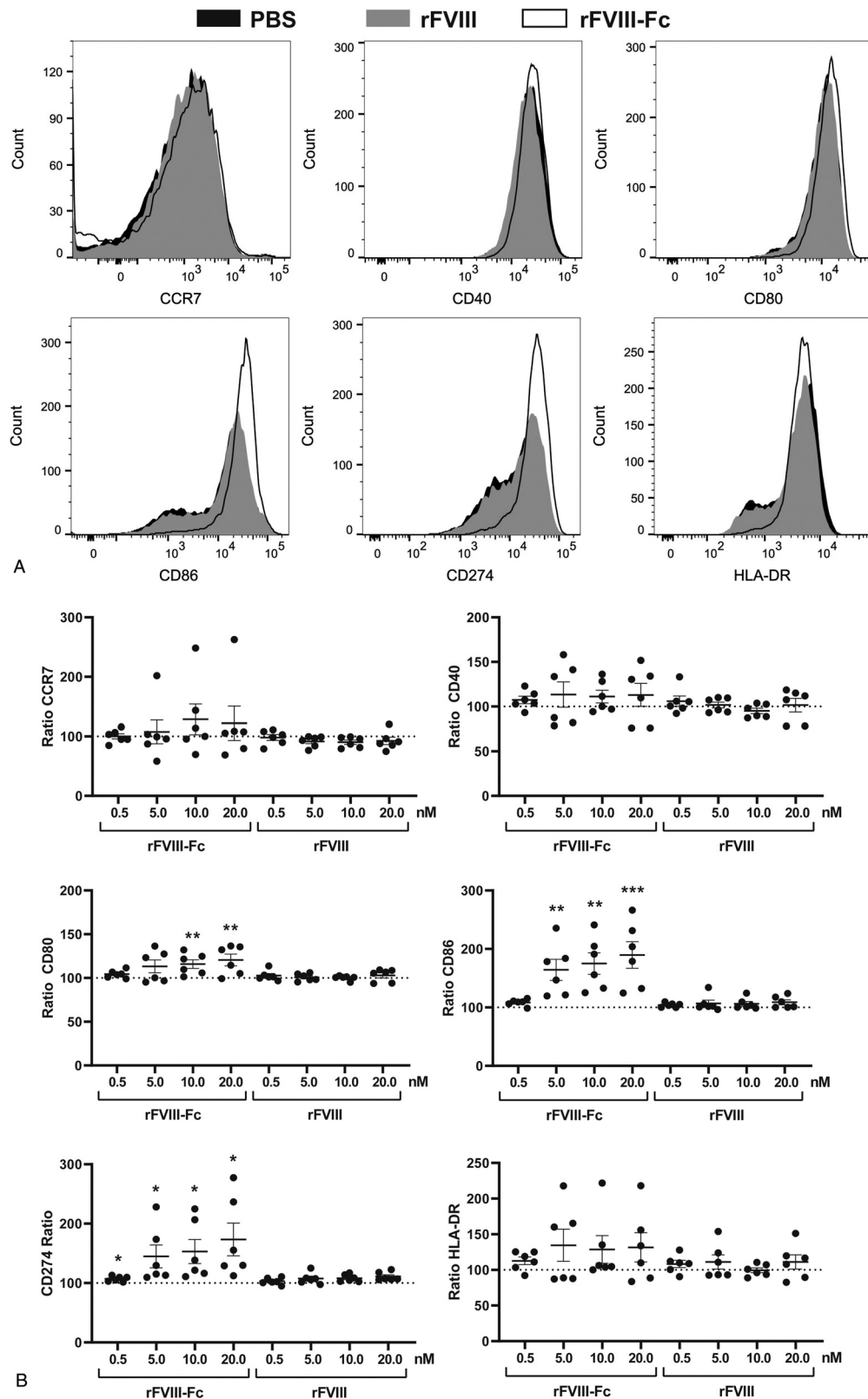


Figure 4. Effect of rFVIII-Fc and rFVIII on the expression of activation markers on moDC's that were additionally stimulated with LPS. moDC's were cultivated with 0.5, 5, 10, and 20 nM rFVIII-Fc or rFVIII for 3 hours and then challenged with 1 μ g/ml LPS for additional 20 hours. PBS-treated cells served as a control. Expression of CCR7, CD40, CD80, CD86, CD274, and HLA-DR was determined by flow cytometry on viable, single cells. (A) Displayed are representative histograms of one donor treated with 10 nM rFVIII-Fc (black line, no filling), rFVIII (grey filled) or PBS (black filled). (B) Summary of the changes in surface expression of moDC's obtained from 6 healthy donors treated with rFVIII-Fc or rFVIII. Data are presented as mean percentage of change in MFI of FVIII-stimulated cells over MFI of PBS-stimulated cells \pm SEM with each dot representing one donor. Data of each concentration were analyzed using one-way ANOVA followed by Dunnett Multiple Comparison test relative to cells treated with PBS (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

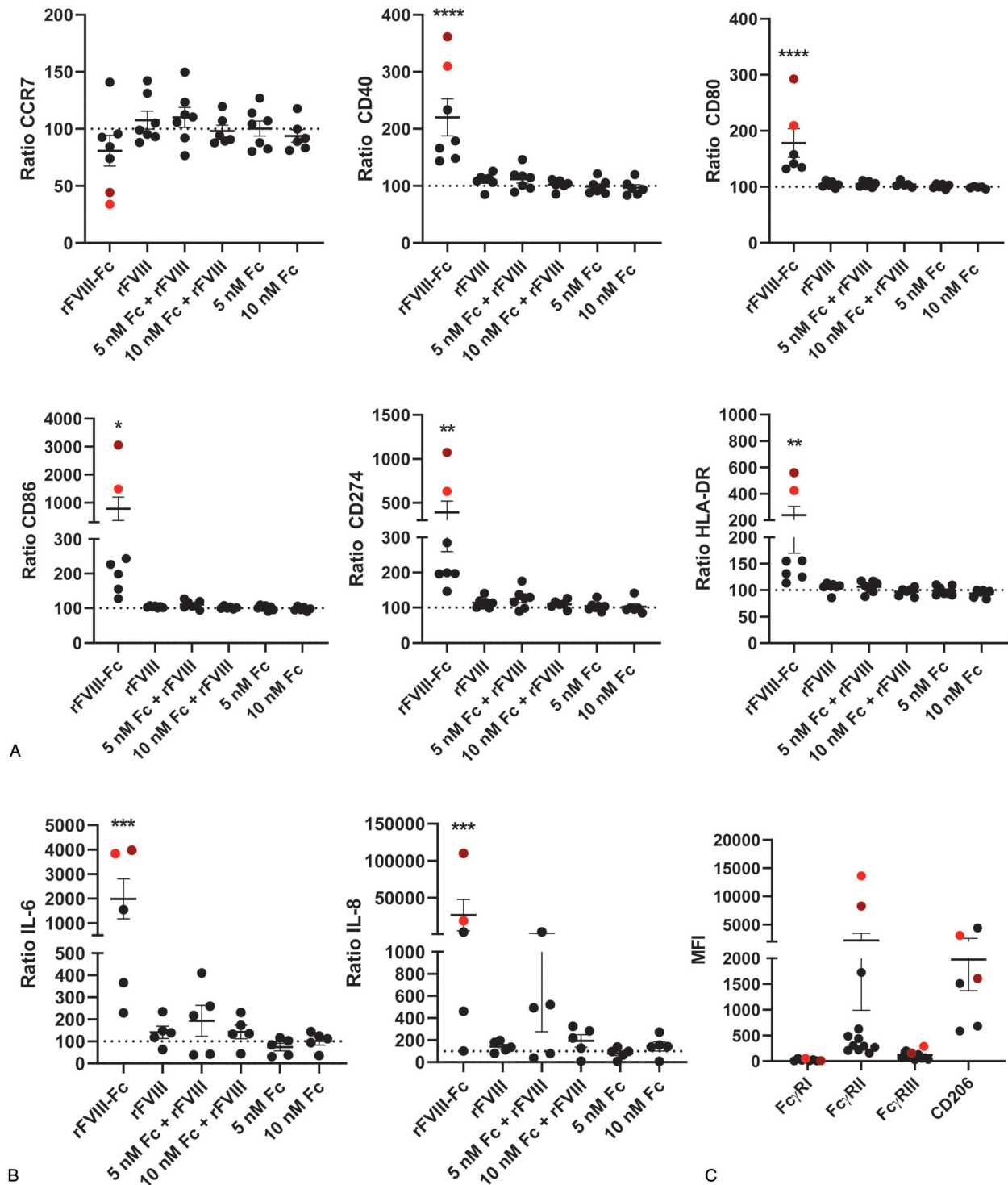


Figure 5. Effect of the Fc domain on moDC activation. moDC's were incubated with 5 or 10nM recombinant human IgG1 Fc in the presence or absence of 5nM rFVIII for 23 hours. 5nM rFVIII-Fc-, 5nM rFVIII-, and PBS-treated cells served as a control. (A) Expression of CCR7, CD40, CD80, CD86, CD274, and HLA-DR was determined by flow cytometry on viable, single cells. Data are presented as mean percentage of change in MFI of FVIII-stimulated cells over MFI of PBS-stimulated cells \pm SEM with each dot representing one donor. (B) IL-6 and IL-8 concentrations were determined simultaneously via cytometric bead array. Data are presented as mean percentage of change in cytokine amount of FVIII-stimulated cells over cytokine amount of PBS-stimulated cells \pm SEM with each dot representing one donor. Data of each concentration were analysed using one-way ANOVA followed by Dunnett Multiple Comparison test relative to cells treated with PBS (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). (C) Expression of Fc γ RI, Fc γ RII, Fc γ RIII, and CD206 on moDC's. Data are presented as mean MFI \pm SEM with each dot representing one donor. Two donors, which strongly increased the expression of surface expression markers and pro-inflammatory cytokines upon cultivation with rFVIII-Fc are highlighted in dark and light red in all diagrams.

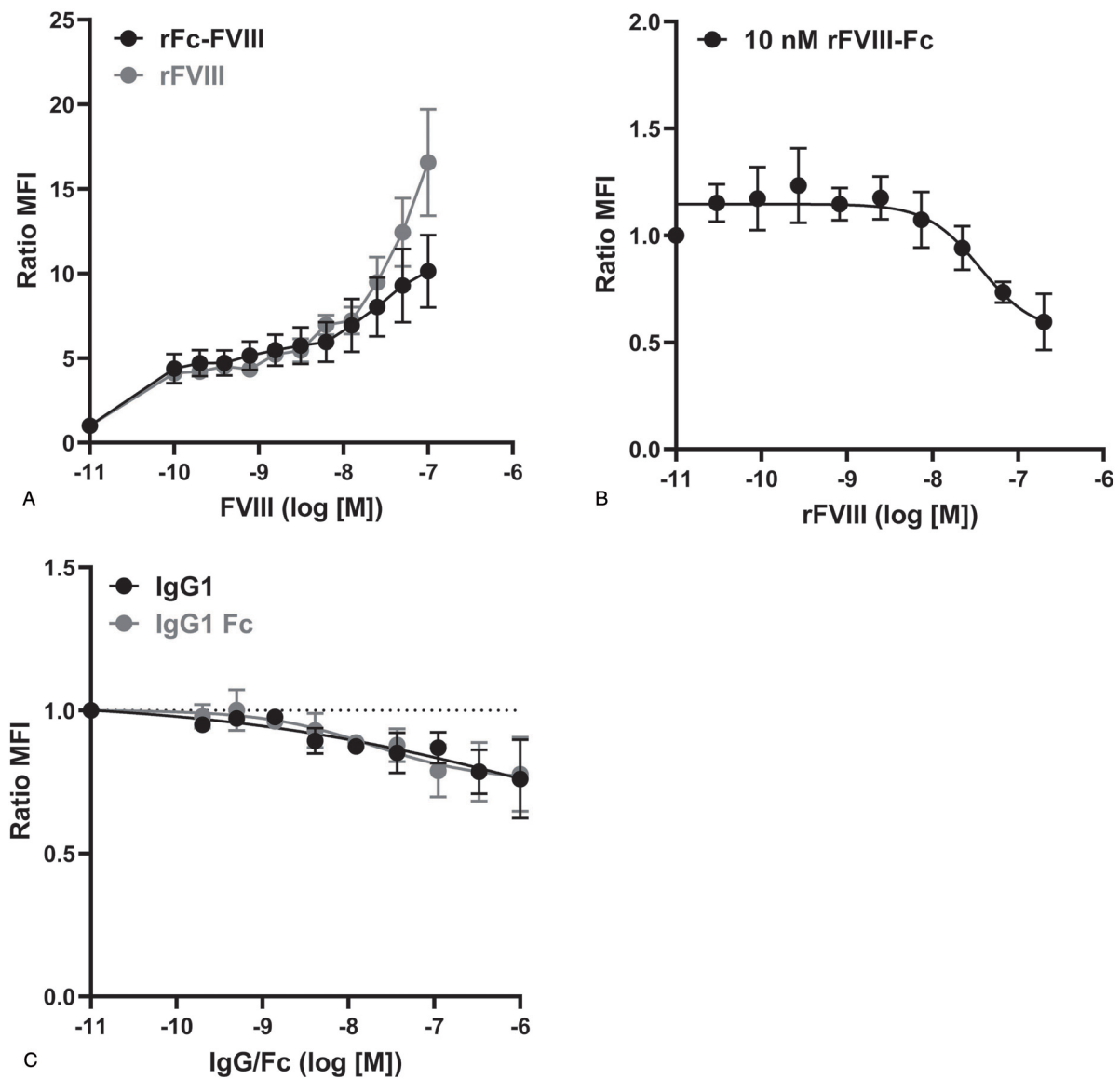


Figure 6. Binding of rFVIII-Fc and rFVIII to moDC's. (A) moDC's were incubated with increasing concentrations of rFVIII-Fc (black line) or rFVIII (grey line) and binding was detected by flow cytometry using a biotin-labeled FVIII-specific nanobody, followed by an incubation with a fluorophore-labeled streptavidin. Data are presented as mean change in MFI of FVIII-treated cells over MFI of buffer-treated cells \pm SEM. (B) Inhibition of rFVIII-Fc binding with increasing concentrations of rFVIII. The 10nM rFVIII-Fc were incubated together rFVIII and binding was detected using APC-coupled F(ab')₂ fragment. Data are presented as mean change in MFI of rFVIII- and rFVIII-Fc-treated cells over MFI of rFVIII-Fc-treated cells \pm SEM. (C) Inhibition of rFVIII-Fc binding with increasing concentrations of human IgG1 (black line) or recombinant human IgG1 Fc (grey line). Binding of 10nM rFVIII-Fc was detected as described in (A). Data are presented as mean change in MFI of rFVIII-Fc and IgG1/Fc-treated cells over MFI of rFVIII-Fc-treated cells \pm SEM. Data are obtained from three experiments with three donors.

that rFVIII-Fc and rFVIII binding is mediated by the same receptor and that Fc γ R2b plays only a minor role in the binding process. Moreover, binding of rFVIII-Fc was only marginally inhibited with increasing concentrations of human monomeric IgG1 or recombinant IgG1 Fc (Fig. 6 (C)).

The binding of rFVIII and rFVIII-Fc appeared to be similar and this raised the question whether rFVIII-Fc is capable of binding to Fc γ R2a. Therefore, binding studies with HEK293 cells transiently transfected with an *FCGR2A*-expression vector were carried out. As shown in Figure 7 (A), rFVIII-Fc efficiently bound to Fc γ R2a-expressing cells. For rFVIII, the binding was low and comparable to non-transfected cells (Fig. 7 (B)). Competition

with an anti-Fc γ R2a antibody showed an efficient inhibition of rFVIII-Fc binding, while much higher concentrations of the isotype control were required to achieve inhibition of binding (Fig. 7 (C)). These findings support the hypothesis that rFVIII-Fc is able to bind to Fc γ R2a.

rFVIII-Fc increases Fc γ R2a phosphorylation

moDC's coexpress the activating (Fc γ R2a) and inhibitory (Fc γ R2b) isoforms of the Fc γ R2.¹⁵ Due to their highly homologous extracellular domains, discrimination between the 2 receptors was not possible. However, since rFVIII-Fc caused

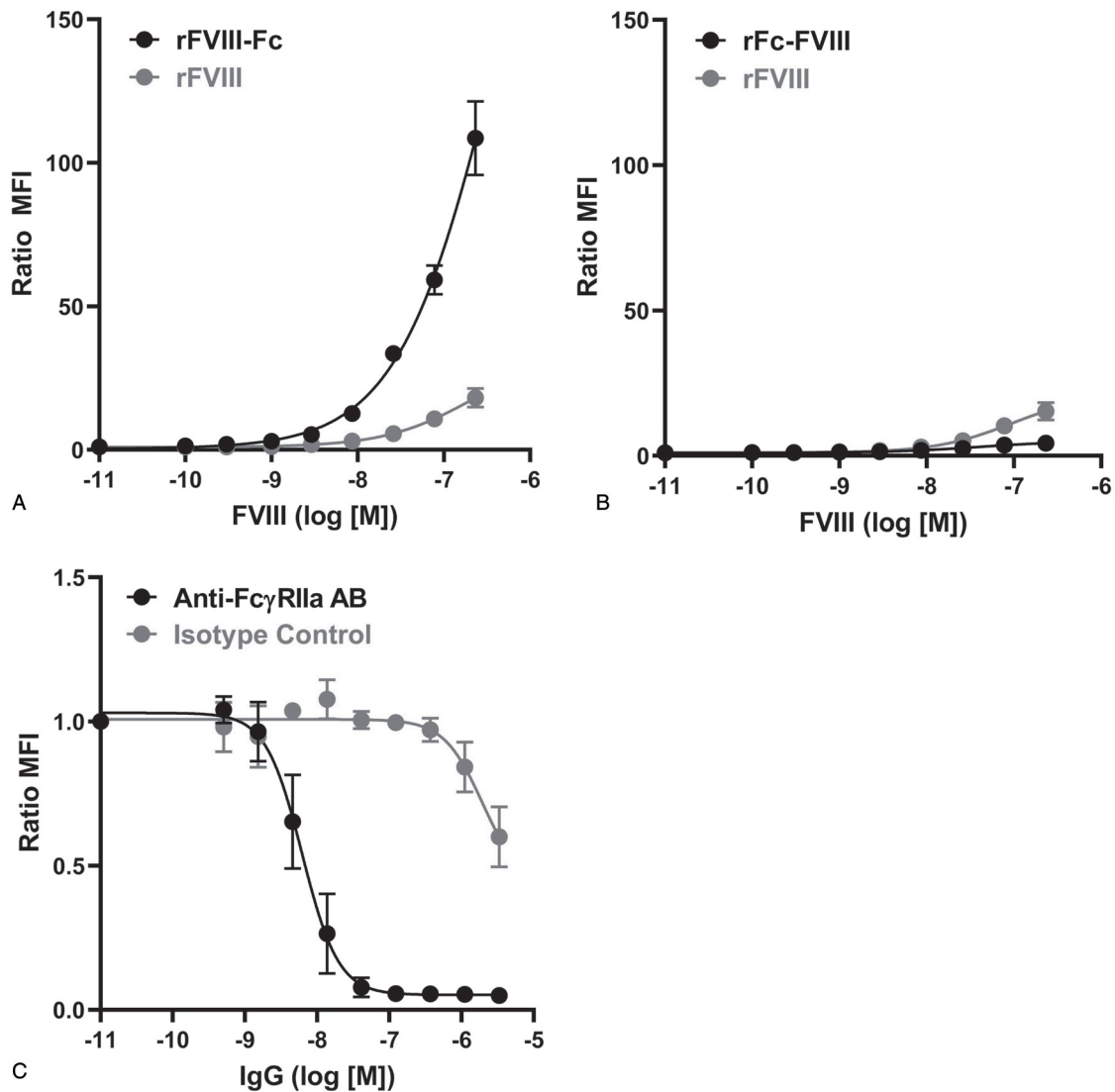


Figure 7. Binding of rFVIII-Fc and rFVIII to FCGR2a-transfected HEK293 cells. HEK293 cells were transiently transfected with a *FCGR2A*-expression plasmid and subjected on the next day to the binding studies. FVIII-binding was detected by flow cytometry using a biotin-labeled FVIII-specific nanobody, followed by an incubation with a fluorophore-labeled streptavidin. (A) *FCGR2A*-transfected cells were incubated with increasing concentrations of rFVIII-Fc (black line) or rFVIII (grey line). (B) Empty vector-transfected HEK293 cells were used as control. Data are presented as mean change in MFI of FVIII-treated cells over MFI of buffer-treated cells \pm SEM obtained from three independent experiments. (C) Inhibition of 10 nM rFVIII-Fc binding with increasing concentrations of FcγRIIa-specific blocking antibody (black line) or an isotype matched antibody control (grey line). Data are presented as mean change in MFI of antibody - and rFVIII-Fc-treated cells over MFI of rFVIII-Fc-treated cells \pm SEM obtained from three independent experiments.

moDC activation, it can be concluded that FcγRIIa is targeted by rFVIII-Fc. Activation of FcγRIIa is mediated by phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic tail.²³ This initiates a cascade of signaling events which results in DC maturation and enhanced expression of pro-inflammatory cytokines.¹⁵ To evaluate if FcγRIIa is targeted by rFVIII-Fc, moDC's were incubated with 5 nM rFVIII-Fc and the phosphorylation of various immune receptors, including FcγRIIa, was analyzed via a phospho-immunoreceptor antibody array. Incubation with rFVIII or PBS served as a control. As shown in Figure 8, increased phosphorylation of FcγRIIa was induced by incubating the cells with rFVIII-Fc, but not with rFVIII or PBS. These findings support the hypothesis of moDC activation by rFVIII-Fc via FcγRIIa.

FcγRIIa blockade reduces rFVIII-Fc-mediated moDC activation

In order to confirm that FcγRIIa in fact provokes activation of moDC's via FcγRIIa, cells were pre-incubated for 1 hour with an FcγRIIa-specific blocking antibody before adding rFVIII-Fc. Cells treated with the same concentration of an isotype-matched antibody served as a control. As shown in Figure 9 (A), incubation with an FcγRIIa-specific blocking antibody prior to cultivation with rFVIII-Fc reduced the cell clustering. Moreover, the FcγRIIa-specific blocking antibody significantly reduced the expression of CD40, CD80, CD86, CD274, and HLA-DR on moDC's and decreased the level of IL-6 and IL-8 in the supernatant (Fig. 9 (B) and (C)). The FcγRIIa-specific blocking

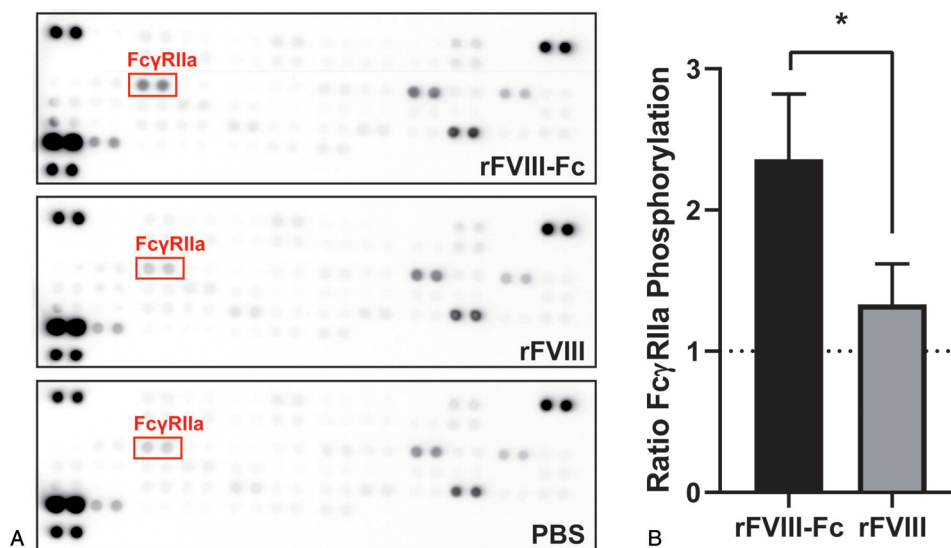


Figure 8. Increased Fc γ RIIa phosphorylation after incubation with rFVIII-Fc. moDC's were incubated for 10 minutes with 5 nM rFVIII-Fc, rFVIII or an equal volume of PBS, lysed and subjected to the human phospho-immunoreceptor antibody array. (A) Displayed are the representative results from one donor treated with the 2 rFVIII products or PBS. The red box highlights Fc γ RIIa phosphorylation. (B) Summary of the results from 3 donors treated with rFVIII and 4 donors treated with rFVIII-Fc. Data are presented as mean ratio of change in raw volume of FVIII-stimulated cells over raw volume of PBS-stimulated cells \pm SEM. Data were analyzed using a one-tailed paired *t* test (**p* \leq 0.05).

antibody resulted in similarly reduced surface marker expression and cytokine levels as observed when the cells were treated with rFVIII. In contrast, pre-incubation of the isotype control antibody with rFVIII-Fc did not affect the rFVIII-Fc-induced cell clustering nor did it alter the expression of the surface markers or cytokine levels. Taken together, these findings strongly support the hypothesis that the observed activation of moDC's by rFVIII-Fc is mediated via Fc γ RIIa signalling.

Discussion

Hemophilia A patients are dependent on the substitution with exogenous FVIII to achieve hemostasis. One of the most serious complications during FVIII replacement therapy is the development of anti-FVIII antibodies, which interfere with the infused FVIII and make replacement therapy ineffective. Thus, a major concern when bioengineering FVIII is to increase the immunogenicity of FVIII. Fusion of the human IgG1 Fc domain to FVIII has been shown to prolong the half-life of the clotting factor. However, the immunological consequences that arise from the Fc fusion are not completely understood. Clinical data show that previously treated patients with severe hemophilia did not develop neutralizing antibodies to rFVIII-Fc.²⁴ However, these patients are less likely to develop inhibitors. Unfortunately, data from clinical trials conducted with previously untreated patients, which would shed more light on the immunogenicity of rFVIII-Fc, are not yet available.

Extrapolating immunological data from other Fc-containing therapeutic proteins appear to be difficult. Among other factors, it depends on the protein that carries the Fc part as well as the applied in vitro or in vivo models. While some publications support the idea that Fc fusion proteins can promote tolerance,⁷ it should be noted that a number of human monoclonal antibodies induce anti-drug antibodies in a substantial number of patients.⁸ Moreover, Fc fusion vaccines have been shown to induce a much stronger antigen-specific immune response compared to vaccines

with the native antigen.^{9,25,26} Thus, careful evaluation of immunological properties of a new Fc fusion construct is crucial.

DC activation and maturation leads to various changes, such as an reduction in antigen uptake, enhanced presentation of antigen-derived peptides by MHC class II, altered expression of chemokine receptors and adhesion molecules involved in migration²⁷ as well as the formation of cell clusters.¹⁷ These functional alterations are tightly linked to the reorganization of the cytoskeleton²⁸ and are believed to be required for the DC's to efficiently present antigens to T cells.²⁹ The in vitro moDC model applied in this study revealed several hallmarks of cell activation following rFVIII-Fc exposure as described above. Cell clustering, up-regulation of co-stimulatory molecules such as CD40 and CD80, as well as increased levels of the pro-inflammatory cytokines IL-6 and IL-8 were exclusively observed after treatment of cells with rFVIII-Fc, but not rFVIII. In contrast to rFVIII-Fc, the mixture of rFVIII and purified IgG1 Fc did not trigger moDC activation.

Although rFVIII-Fc treatment predominantly induced signals typical of cell activation and maturation, reduced levels of the cytokine receptor CCR7 and increased levels of the inhibitory receptor ligand CD274 were detected as well. CCR7 is implicated in migratory activity of DC's and a reduction in this receptor might have functional consequences with respect to the homing of DC's to the lymphoid organs. While the increased expression of CD40, CD80, CD86 and HLA-DR following rFVIII-Fc treatment provides a strong stimulatory signal for T-cell activation, an increased expression of CD274 (also called programmed death-ligand 1 or PD-L1) on DC's transmits an inhibitory signal to T cells via PD-1.³⁰ Engagement of CD274 with PD-1 on T cells can block T cell proliferation, cytokine production and inhibit T cell survival.³¹ However, this inhibitory signal can be overturned by CD28 co-stimulation or IL-2.^{32,33} Moreover, up-regulation of CD274 has also been observed following stimulation with pro-inflammatory cytokines in various cell types,^{34,35} and appears to be a regulatory feedback mechanism to limit the intensity of DC

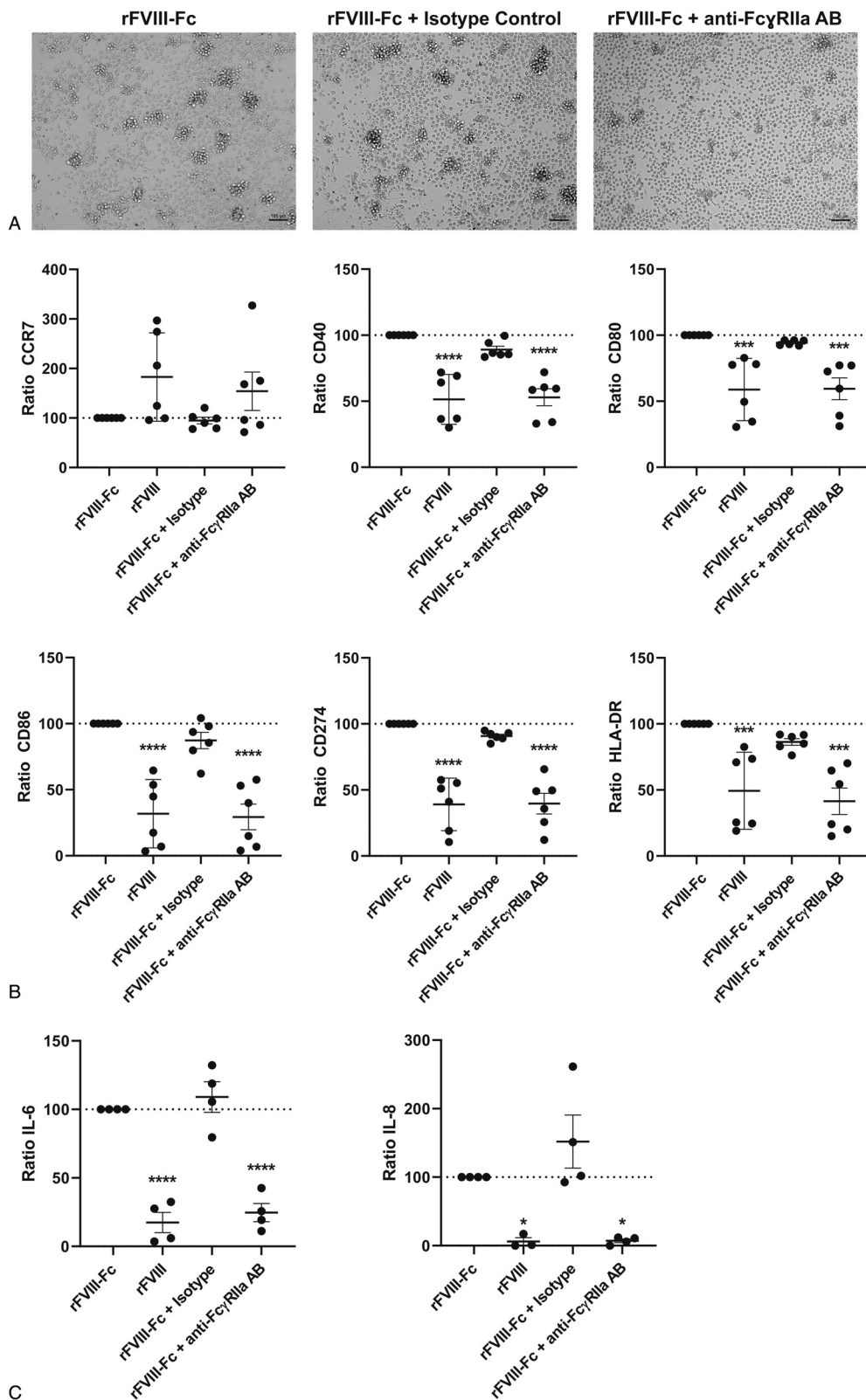


Figure 9. FcγRIIa-specific blocking antibody reduces rFVIII-Fc-mediated cell clustering, moDC activation and levels of IL-6 and IL-8. moDC's were pre-incubated with 1 mg/ml FcγRIIa-specific blocking antibody (anti-FcγRIIa AB) or an isotype matched antibody (Isotype) for 1 hour followed by a further incubation for 23 hours with 5 nM rFVIII-Fc. Cells treated only with 5 nM rFVIII-Fc or rFVIII served as a control. (A) Morphological changes were documented 23 hours post treatment with the Axio Observer Z1 microscope using a 100-fold magnification. Displayed are images from one representative donor. (B) Expression of CCR7, CD40, CD80, CD86, CD274, and HLA-DR was determined by flow cytometry on viable, single cells. Data are presented as mean percentage of change in MFI of antibody pre-incubated cells over MFI of rFVIII-Fc-stimulated cells ± SEM, with each dot representing one donor. (C) IL-6 and IL-8 concentrations in the supernatant of the treated moDC's were determined simultaneously via cytometric bead array. Data are presented as mean percentage of change in cytokine concentration of antibody pre-incubated cells over cytokine concentration of rFVIII-Fc-stimulated cells ± SEM, with each dot representing one donor. Data were analyzed using one-way ANOVA followed by Dunnett's Multiple Comparison test relative to cells treated with rFVIII-Fc only (*p ≤ 0.05, ***p ≤ 0.001, ****p ≤ 0.0001).

activation. In line with this, an up-regulation of CD274 following LPS stimulation after 23 hours was observed (SDC, Fig. 2, <http://links.lww.com/HS/A58>), while all other co-stimulatory molecules as well as HLA-DR were upregulated. Thus, the up-regulation of CD274 following rFVIII-Fc treatment probably reflects a common physiological regulatory feedback mechanism that follows a strong stimulation event rather than indicating an overall inhibitory effect of rFVIII-Fc.

Although a dose-dependent increase in the expression of the surface markers was detected following rFVIII-Fc cultivation, it appears that rFVIII-Fc did not activate all moDC's (SDC, Figure 3, <http://links.lww.com/HS/A58>). Especially for the expression of CD86 and HLA-DR, two populations appeared in the histogram following rFVIII-Fc stimulation. When 5 nM or more of rFVIII-Fc were applied to the cells, more than 40% of the living cells were activated, that is, high in CD86 and HLA-DR expression. The high expression of CD86 correlated with the high expression of HLA-DR, showing that these are the same cells. Activation of all cells might be achieved by using higher concentrations of rFVIII-Fc.

The observation that rFVIII-Fc causes the activation of moDC's is in part contradictory to previous results from 2 other studies. Krishnamoorthy et al investigated the immune response of rFVIII-Fc in FVIII-deficient mice.³⁶ The immune response following rFVIII-Fc treatment appeared to be strongly concentration-dependent in this model. At 50 and 100 IU/kg rFVIII-Fc fewer total anti-FVIII antibodies were detected compared to a treatment with recombinant FVIII lacking the Fc domain. Moreover, rFVIII-Fc appeared to induce the expression of molecules involved in immune suppression or tolerance induction, including an increased expression of CD274 on murine DC, which is in agreement with our results. By contrast, treatment with 250 IU/kg rFVIII-Fc resulted in increased levels of non-neutralizing and neutralizing anti-FVIII antibodies and significantly boosted T cell proliferation and IFN- γ production. It is important to note, that the source of study material was different in the study by Krishnamoorthy: Human embryonic kidney cell-derived rFVIII-Fc was compared to rFVIII products produced in Chinese hamster ovary cells. Both expression systems differ in the nature of their post-translational modifications, which might have an impact on their potential to induce an immune response. Moreover, the applied hemophilia A mice model is intrinsically limited with respect to evaluating Fc fusion constructs, as Fc receptors differ between humans and mice. Thus, certain immune responses triggered by the human Fc domain might not be detected in the murine model. In the other rFVIII-Fc-related study by Kis-Toth using human monocyte-derived macrophages,³⁷ rFVIII-Fc induced phenotypical alteration, which the authors interpreted as regulatory macrophage polarization. However, it is worth noting that these phenotypic alterations were achieved following treatment with a very high concentration of rFVIII-Fc (200 nM or 400 U/ml), which is more than 100 times higher than the naturally occurring concentration.

The findings presented here give evidence that the observed activating effect of rFVIII-Fc is mediated by the interaction with Fc γ RIIa, which is expressed together with Fc γ RIIIb on moDC's.¹⁵ Blockade of Fc γ RIIa almost completely abolished the effect of rFVIII-Fc. Moreover, a rapid increase of Fc γ RIIa phosphorylation was detected upon incubation with rFVIII-Fc, which supports the hypothesis that rFVIII-Fc is capable of activating Fc γ RIIa signaling. In line with this, two donors with significantly increased Fc γ RII expression levels showed stronger activation signals upon rFVIII-Fc incubation.

The ability of rFVIII-Fc to bind to Fc γ RIIa was shown with FCGR2A-transfected HEK293 cells. Here rFVIII-Fc bound efficiently to the transfected cells, while the binding of rFVIII was low and comparable between FCGR2A-transfected and empty vector-transfected HEK293 cells. Interestingly, on moDC's the binding pattern did not differ between rFVIII-Fc and rFVIII, indicating that Fc γ RIIa does not play an important role in the overall rFVIII-Fc binding. This is not surprising, since Fc γ RIIa has a low affinity to monomeric IgG's and binds only multimeric or aggregated IgG efficiently. The applied rFVIII-Fc and rFVIII preparations contained only a very low percentage of aggregates and the ratio of FVIII activity to FVIII antigen was comparably high for both recombinant FVIII products (SDC, Fig. 4, <http://links.lww.com/HS/A58>). Therefore, main FVIII binding is most likely mediated by FVIII-binding receptors. This is supported by the finding that rFVIII-Fc binding is efficiently inhibited with rFVIII and only high concentrations of human IgG1 or Fc are capable of reducing rFVIII-Fc binding (Fig. 6).

Several receptors which are involved in FVIII binding, uptake and immune-recognition by DC's have been identified^{22,38} and could, together with Fc γ RIIa, be responsible for the observed cell activation. Potential interaction partners that are present on moDC's are LRP1, CD206, Siglec-5,³⁹ galectin-1, and galectin-3.⁴⁰ LRP1 binds FVIII with moderate affinity and was shown to be involved in the clearance of FVIII in mice.⁴¹ However, in a human in vitro model LRP1 does not seem to play a role in FVIII uptake and activation of T cells.^{21,42} The impact of CD206 on FVIII uptake and immunogenicity in moDC has been a matter of debate: While in one study saturating and blocking CD206 reduced the uptake of FVIII in moDC's and decreased T-cell activation,²⁰ no inhibitory effect on FVIII binding and uptake was detected in another study.²¹ Similar to the latter results, we did not observe a reduction of rFVIII-Fc or rFVIII binding if the cells were pre-incubated with different concentrations of mannan (SDC, Fig. 5, <http://links.lww.com/HS/A58>). Amongst the FVIII-binding lectins, galectin-1 and -3 appear to have the highest affinity towards FVIII.⁴⁰ However, whether these lectins are involved in FVIII uptake and the extent to which they contribute to FVIII immunogenicity remains to be established.

moDC activation relies on the presence of the rFVIII-Fc fusion construct. Neither rFVIII or IgG1 Fc alone, nor rFVIII together with IgG1 Fc (applied as 2 separate proteins) have a comparable activating effect on moDC's. Moreover, the addition of human IgG1 Fc together with rFVIII-Fc is also not capable of blocking the rFVIII-Fc-induced cell activation (SDC, Fig. 6, <http://links.lww.com/HS/A58>). Thus, we speculate that close proximity of rFVIII and Fc, as it is achieved in the fusion construct, is required to cluster FVIII receptors together with Fc γ RIIa. This initiates cellular signaling cascades that lead to moDC activation and maturation. It has been assumed that ligand binding to members of the FC γ R family leads to the activation of Src-family kinases, which in turn phosphorylate the ITAM motif present in the cytosolic domain of the receptor. The phosphorylation creates a docking site for the kinase Syk, which then phosphorylates down-stream substrates, leading to the initiation of various signaling pathways and cell activation.^{13,43} Interestingly, Src- and Syk-initiated signaling cascades following immune-receptor stimulation fed into the Rho/WASP pathway, which plays an important role in modulating the structure of the actin cytoskeleton.⁴⁴ It is tempting to speculate that the observed cell-clustering process is rFVIII-Fc-driven and functions *via* the Src- and Syk-initiated signaling pathways.

MoDC's express both activating and inhibitory Fc γ RII's, which possess opposing functions due to the presence of the

cytosolic immunoreceptor tyrosine-based activating or inhibitory motif. While ligation of FcγRIIa on DC's was shown to induce cell maturation, increase stimulation of T cells and elevate the expression of pro-inflammatory cytokines, binding to FcγRIIb rather inhibits the immune response.¹⁵ Based on the data presented here, rFVIII-Fc seems to specifically target FcγRIIa and not or at least to a lesser extent FcγRIIb. Given the structural similarity of the extracellular domain of the two FcγRII with 89% sequence identity, the question remains: Why does rFVIII-Fc signal via FcγRIIa? Affinity and preference towards these highly related receptors are not well understood. However, one factor that greatly influences the binding to Fc receptors, and thus the biological activity of the Fc domain, is its glycosylation. For example, terminal sialylation of the Fc domain has been shown to be important for the anti-inflammatory activity of IgG's,^{45,46} probably by promoting an anti-inflammatory milieu through the interaction with sialic acid recognizing receptors.⁴⁷ Interestingly, Kannicht et al showed that the Fc domain of rFVIII-Fc contains a remarkably low level of sialylation (less than 1%).⁴⁸ In contrast, the sialylation degree of the Fc domain of plasma-derived human IgG's is around 13%.⁴⁹ Thus, it can be speculated, that the very low sialic acid content of the fusion construct (and the abolishment of binding to sialic acid-specific receptors) in combination with the binding to FVIII-specific receptors and a co-ligation with FcγRIIa supports signaling pathways that drive DC activation.

The in vitro model of human moDC's applied in this study has been extensively used in basic research to study DC biology and was implemented successfully in numerous clinical trials.⁵⁰ While the ready availability of large quantities of cells is a big advantage of this model, it should be kept in mind that moDC's differ in terms of function from in vivo-occurring human DC subsets. In general, moDC's share many features of DC's following stimulation: They are capable of capturing, processing and presenting antigens to T cells and are able to produce important chemokines and cytokines.⁵¹⁻⁵³ However, recent evidence suggests that moDC's are rather inflammatory monocytes with DC-like functions.^{54,55} Thus, translation to human DC's in vivo should be made with caution. Analysis of naturally in vivo-occurring DC subsets, such as plasmacytoid or conventional DC's would be of great value to ascertain whether rFVIII-Fc behaves similarly to the moDC's.

Here we report for the first time that rFVIII-Fc leads to activation of moDC's at therapeutically relevant doses in vitro. Mechanistically, this effect appears to be mediated by FcγRIIa. Although two other studies report tolerogenic properties of rFVIII-Fc, the presented interim study with previously untreated patients⁵⁶ did not show lower rates of inhibitor development in this patient group compared to conventional rFVIII products, indicating that rFVIII-Fc is not tolerogenic per se. Moreover, studies with hemophilia mice, which do not express the genetic equivalent of the human FcγRIIa,¹³ might underestimate the unwanted immunological consequences caused by the Fc fusion construct. Thus, careful investigations in appropriate in vivo and in vitro models are required to better understand the immunogenicity of Fc fusion proteins.

Materials and methods

Materials

Two batches of the 2 different recombinant B-domain deleted FVIII product, rFVIII-Fc (Elocta[®], 2000 Units, Sobi) and rFVIII

(Nuwiq[®], 2000 Units, Octapharma), were dissolved in sterile water according to the manufacturers recommendations, aliquoted and stored at -80°C until further usage. FVIII activity and antigen concentration was determined by chromogenic FVIII assay (Chromogenix) and FVIII antigen ELISA (ASSERACHROM VIII:Ag, Stago), respectively. Plasmatic human IgG1 was obtained from Athens Research & Technology, recombinant human IgG1 Fc (Thr106-Lys330, 293E expressed) from Biologend. Fluorophore-coupled monoclonal antibodies against human HLA-DR (Clone L234), CD40 (Clone 5C3), CD86 (Clone BU63), CCR7 (Clone G043H7), CD274 (Clone 29E.2A3), FcγRI (Clone 10.1), FcγRII (Clone FUN-2), FcγRIII (Clone 3G8), CD206 (Clone 15-2), APC-streptavidin were purchased from Biologend and against human CD80 (Clone L397.4) from BD. APC-coupled F(ab')₂ fragment specific for human IgG Fc-part was purchased from Jackson Immuno Research. Tag-free expression vector containing the sequence of human FCGR2A (NM_001136219) as well as an empty control vector (pCMV6-XL5) were purchased from OriGene.

Monocyte purification, moDC stimulation, and flow cytometric analysis

Monocytes from healthy donors, obtained from buffy coats from the DRK-Blutspendedienst Ost, were enriched via Ficoll (GE Healthcare) gradient and subsequently CD14+ monocytes were purified by magnetic cell sorting (CD14+ monocyte isolation Kit, Miltenyi Biotec). The purity of the cells was measured by flow cytometry and was always more than 85% of the living cells. To obtain immature moDC's, CD14+ monocytes were cultivated for 5 to 6 days in RPMI medium supplemented with 10% fetal calf serum (FCS, Biochrome), 100 U/ml penicillin, 100 µg/ml streptomycin, 100 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF, R&D) and 200 U/ml interleukin-4 (IL-4, R&D). In order to analyze the effect of the FVIII products on cell maturation, 2.5x10⁵ moDC's per well of 48-well plate were incubated for 23 hours with indicated concentrations of FVIII. Cells treated with the same volume of phosphate buffered saline (PBS) served as a control. Morphological alterations of the cells following rFVIII-Fc, rFVIII or PBS treatment were documented with the Axio Observer Z1 microscope (Zeiss) using a 100-fold magnification. Since the micro-environment can affect the immunogenicity of a foreign protein, the impact of the FVIII products in the presence of LPS was investigated. Therefore, moDC's were pre-incubated for 3 hours with different concentrations of FVIII and then stimulated with 1 µg/ml LPS from *P aeruginosa* serotype 10 (Sigma) for 20 hours. Expression of surface markers involved in T cell stimulation (CCR7, CD40, CD80, CD86, CD274, and HLA-DR) was determined subsequently by flow cytometry. In order to detect fluorescence signals obtained from unspecific binding of the antibodies to dead cells, cells were treated with Zombie Aqua dye (Biologend). Samples were analyzed with the flow cytometer FACSVerse (BD). Final examination of the data was carried out using FlowJo software (Tree Star, Inc.). To normalize for variability between the different donors, expression of surface receptors was expressed as the ratio of the median fluorescence intensity (MFI) by dividing the MFI of FVIII-incubated cells by the MFI of cells incubated with the same volume of PBS.

In parallel to the quantification of surface markers, the cytokine profile secreted by the moDC's was analyzed 23 hours post stimulation following supernatant collection via the cytometric bead array CBA Flex (BD) detecting simultaneously

interleukin-6 (IL-6) and interleukin-8 (IL-8) according to the manufacturer's recommendation. Samples were analyzed with the flow cytometer FACSVerse (BD). Final analysis and calculation of the cytokine concentration were carried out using FCAP Array software (BD).

Binding experiments

Binding experiments were carried out with moDC's or transiently-transfected HEK293 cells. For expression of human FcγRIIa, 2×10^6 HEK293 cells were seeded in a 10 cm dish in Roti-CELL DMEM High Glucose (Roth) supplemented with 5% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. On the following day, cells were transfected using Lipofectamine 2000 Transfection Reagent according to the manufacturer's recommendation with 10 μg of the expression vector for FcγRIIa or empty vector. On the next day, successful transfection was verified by flow cytometry and cells were subjected to binding studies.

For rFVIII binding studies, 2×10^5 moDC's or transfected HEK293 cells per test were washed with FACS-buffer (PBS containing 1% FCS) and incubated with various concentrations of rFVIII-Fc or rFVIII for 30 minutes on ice. Following two wash steps with FACS buffer, cells were incubated with the CaptureSelect Biotin Anti-FVIII Conjugate (ThermoFisher Scientific) for 30 minutes on ice. Unbound detection reagent was removed by washing once with FACS buffer. For detecting bound rFVIII, APC streptavidin was applied to the cells for 30 minutes on ice, followed by two wash steps with FACS buffer. MFI of APC was quantified via flow cytometry. For binding inhibition experiments, the cells were pre-treated for 10 minutes on ice with various concentrations of human IgG1 or recombinant human IgG1 Fc, followed by the addition of 10 nM rFVIII-Fc for further 30 minutes on ice in the first step. Detection of bound rFVIII-Fc was carried out as described above.

For competition binding experiments, 2×10^5 moDC's per test were washed with FACS-buffer and pre-treated with various concentrations of rFVIII for 10 minutes on ice, followed by the addition of 10 nM rFVIII-Fc and an incubation for 30 minutes on ice. After two wash steps with FACS buffer, rFVIII-Fc-treated cells were incubated with Fc-specific APC AffiniPure F(ab')₂ fragment for 30 minutes on ice. Unbound streptavidin was removed by washing with FACS buffer. Binding of rFVIII-Fc was determined by measuring the MFI of APC via flow cytometry. Final examination of the data was carried out using FlowJo software.

Human phospho-immunoreceptor array

For determining the influence of FVIII on tyrosine phosphorylation of human immunoreceptors, moDC's were incubated for 10 minutes with 5 nM rFVIII-Fc or rFVIII and then lysed. PBS-treated cells served as a control. Protein concentration was determined via BCA (Pierce) and the Proteome Profiler Human Phospho-Immunoreceptor Array (R&D systems) was carried out according to the manufacturer's recommendations using 500 to 1000 μg cell lysate. Images of the membranes were captured using GeneSys imaging software on a G:BOX Chemi system. Raw volume of the spots was determined using GeneTools analysis software (Syngene) and fold change of raw volume of FVIII-stimulated cells over raw volume of PBS-stimulated cells was calculated using Excel.

FcγRIIa-specific blocking experiment

2.5×10^5 moDC's per well of 48-well plate were pre-incubated with 1 mg/ml FcγRIIa-specific blocking antibody (clone IV.3, Hölzel Biotech) or with the same concentration of LEAF™ Purified Mouse IgG2b, κ Isotype control antibody (Biolegend). After 1 hour, 5 nM rFVIII-Fc were added and the cells were incubated for another 23 hours. Cells not treated with the blocking antibody or the isotype control antibody served as a control. Analysis of surface marker expression and cytokine levels were carried out as described above.

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