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ORIGINAL ARTICLE

Development of a novel fully functional coagulation factor VIII with reduced immunogenicity utilizing an *in silico* prediction and deimmunization approach

Karina Winterling[1] | William D. Martin[2] | Anne S. De Groot[2,3] | Jens Daufenbach[1] | Steffen Kistner[1] | Jörg Schüttrumpf[1]

[1]Biotest AG, Dreieich, Germany

[2]EpiVax, Inc., Providence, Rhode Island, USA

[3]Center for Vaccines and Immunology, University of Georgia, Athens, Georgia, USA

Correspondence
Steffen Kistner, Biotest AG, Landsteinerstrasse 5, 63303 Dreieich, Germany.
Email: Steffen.Kistner@biotest.com

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Biotest AG

Abstract

Background: Up to 30% of hemophilia A patients develop inhibitory antibodies against the infused factor VIII (FVIII). The development of a deimmunized FVIII is an unmet high medical need. Although improved recombinant FVIII (rFVIII) products evolved within the last years, the immunogenicity has not been solved. A deimmunized FVIII could reduce the probability of inhibitor development, providing safer therapy.

Objective: To develop a deimmunized FVIII molecule by modifying major histocompatibility complex (MHC) class II presentation, leading to a functional but less immunogenic molecule.

Methods: We performed (1) *in silico* prediction of potentially immunogenic T cell epitopes and their modification by amino acid substitutions in the FVIII sequence, (2) evaluation of functional and structural similarity of the modified rFVIII to unmodified FVIII and registered products, and (3) confirmation of the reduced immunogenicity by *in vitro* testing.

Results: A partially deimmunized fully functional FVIII molecule incorporating 19 amino acid substitutions was generated. The substitutions led to a reduction of the immunogenicity score, indicating a reduced immunogenicity based on *in silico* calculations. This was confirmed in an *in vitro* dendritic cell (DC)--T cell assay. Using this assay, cells from healthy donors proved the significantly reduced immunogenicity of the modified FVIII variant by revealing less proliferation of T helper cells to this variant than to the unmodified FVIII.

Conclusion: *In silico* predictions resulted in a partially deimmunized FVIII. This FVIII is fully functional and was demonstrated to be less immunogenic in *in vitro* testing. This approach may result in a reduction of the inhibitor risk for patients with hemophilia A.

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KEYWORDS
blood coagulation factor inhibitors, factor VIII, hemophilia A, histocompatibility antigens class II, immune system

1 | INTRODUCTION

Hemophilia A is a genetic bleeding disorder caused by the absence of sufficient amounts of active factor VIII (FVIII).[1] A major drawback in therapy is the development of inhibitory antibodies. These arise when the immune system recognizes infused FVIII as a foreign protein.[2] Mainly patients with severe hemophilia A, but also patients with mild or moderate forms, develop inhibitors.[3,4] In the case of inhibitor formation, patients often undergo an immune-tolerance-induction (ITI) therapy. This therapy is very cost intensive as well as strenuous for the patients and their caregivers. To address this unmet medical need, the development of a deimmunized recombinant FVIII (rFVIII), which could reduce the risk of an immune response in patients, is encouraged. In this article we explore an approach in which highly immunogenic T cell epitopes are identified and deimmunized by alteration of the amino acid sequence based on *in silico* immune prediction. Thus, FVIII epitopes would be less likely to be presented on the surface of antigen-presenting cells (APCs) due to reduced binding to the major histocompatibility complex (MHC) class II. Lower or absent binding results in a reduced capacity to activate T cells. Due to the reduction in the number of FVIII-specific T helper cells, the ability of B cells to differentiate and produce high-affinity inhibitory antibodies against FVIII would be reduced.[5] In general, deimmunization based on *in silico* analyses is an established approach that has been already successfully performed for other proteins and antibodies like erythropoietin,[6] recombinant interferon α,[7] or emicizumab.[8] In this work we apply this approach for FVIII.

Essentials

- A deimmunized factor VIII (FVIII) may reduce inhibitory antibody development in patients.
- Introduction of amino acid substitutions can remove T cell epitopes and reduce immunogenicity.
- Incorporation of 19 amino acid substitutions leads to a functional FVIII.
- The partially deimmunized FVIII reveals reduced immunogenicity *in vitro*.

2 | METHODS

2.1 | *In silico* analyses

The applied *in silico* tools have been developed and performed by EpiMatrix (EpiVax, Inc.) and have been described in detail elsewhere.[9] In essence, the EpiMatrix tool split the protein into overlapping peptides, comprising nine amino acids. The binding capacity of all nine-mers was then calculated for the eight common MHC class II supertype alleles, representing >98% of the human population.[10] Overlapping nine-mers that bind to MHC class II were clustered using the program ClustiMer.[9] Before the optimization, an analysis regarding similarity of each epitope with endogenous epitopes was performed using the program JanusMatrix.[11] Conservation of the FVIII epitope with at least two endogenous protein epitopes led to the exclusion of a cluster from further modification, as central tolerance could be expected. In addition, clusters that contained critical FVIII cleavage sites, activation sites, or other sites important for the activity of FVIII were not altered. The amino acid substitutions were calculated using OptiMatrix.[9] The proposed substitutions had to be conserved in other species and not registered in the database comprising all known FVIII substitutions leading to hemophilia A.[12]

The EpiMatrix tool was also used to calculate the overall immunogenicity score for a protein. To be able to compare different proteins, the overall score is normalized for the length of the protein (given per 1000 nine-mers) and compared to the scores for a set of 100,000 randomized protein sequences, whose median score is normalized to 0. This overall score is set to reflect the potential for immunogenicity in the general human population.[10] Alternatively, the immunogenicity score can be calculated for a specific human leukocyte antigen (HLA)-DR genotype. This is the "individual T cell epitope measure" (iTEM) score.[13]

2.2 | Production and purification of deimmunized rFVIII variants

The DNA sequence for a partially B-domain-deleted (BDD) FVIII molecule, comprising FVIII amino acid 20 to 763 and 1582 to 2351, was used as the starting point for deimmunization. DNA sequences containing various combinations of the suggested amino acid substitutions were designed using Vector NTI (Thermo Fisher Scientific). In addition, a reference molecule, unmodified FVIII, with the same backbone but without amino acid substitutions was designed. The synthesis of the vectors was performed by Invitrogen GeneArt (Thermo Fisher Scientific).

The vector backbone comprises the human elongation factor-1 alpha (EF-1α) promotor for transcription of the FVIII gene. Additionally, the vector is encoding for ampicillin resistance and dihydrofolate reductase (dhfr), as pro- and eukaryotic selection markers, as well as the pBR322 origin and the simian vacuolating virus 40 (SV40) as origins of replication in pro- and eukaryotes.

Expression of FVIII variants for the deimmunization screening was performed using HEK 293-F cells (Thermo Fisher Scientific), whereas large-scale production for the functional and structural analyses was

performed using CAP-T cells (Cevec Pharmaceuticals GmbH). Both cell types were transfected using the 4D Nucleofector system (Lonza Group Ltd). Cultivation of transfected HEK 293-F cells was performed in CD293 medium supplemented with 4 mM GlutaMAX and 12% HyClone Cell Boost 5 (all Thermo Fisher Scientific). Cultivation of transfected CAP-T cells was performed in protein expression medium (Thermo Fisher Scientific). Both cell types were cultivated for 4 days at 37°C and the FVIII-containing supernatant was harvested by centrifugation.

Purification of the FVIII variants from the cell culture supernatant was performed by chromatography. Subsequent steps of strong anion exchange chromatography (HiTrap Capto Q column), affinity chromatography (VIIISelect column), and size exclusion chromatography (HiTrap Desalting column) were performed (all GE Healthcare).

2.3 | Functional analyses

The activity of the FVIII variants was determined using the chromogenic Coatest SP Factor VIII Kit (Chromogenix, Instrumentation Laboratory) on a BCS XP (Siemens Healthcare GmbH). The amount of FVIII antigen was determined using the Asserachrom VIII:Ag ELISA (Diagnostica Stago).

The clotting time was determined via thromboelastometry (TEM) using the ROTEM system (Werfen). The reagents star-tem and in-tem (Werfen) were used.

Moroctocog alfa (Pfizer) and simoctocog alfa (Octapharma AG) were used as reference BDD rFVIII molecules. In all assays described above the samples were measured in duplicate.

2.4 | Structural analyses

The structure of the FVIII variants was analyzed via sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blot. The heavy chains were detected with the rabbit anti-human FVIII antibody (Sino Biological Inc.) and the donkey anti-rabbit IgG IRDye 800CW (Abm). The light chains were detected with the mouse anti-human FVIII antibody (Merck KGaA) and the donkey anti-mouse IgG IRDye 680RD (Li-Cor Biotechnology GmbH).

Thrombin activation was shown via western blot with the polyclonal sheep anti-human FVIII antibody (Cedarlane) and the donkey anti-sheep IgG IRDye 800CW (Abm). For the thrombin activation prior to the SDS-PAGE, the FVIII samples and thrombin (Sigma-Aldrich Chemie GmbH) were mixed and incubated for 8 min at 37°C. To stop the reaction, lithium dodecyl sulfate-containing buffer was added and incubated for 10 min at 70°C.

2.5 | Dendritic cell-T cell assay

For the dendritic cell (DC)-T cell assay, DCs and $CD4^+CD25^-$ T cells were co-cultivated in the presence of different FVIII variants. Both cell types were purified from cryopreserved peripheral blood mononuclear cell, derived from whole blood donations (Bio-Rad Medical Diagnostics GmbH) or leukapheresis donations (DRK-Blutspendedienst). Monocytes were purified using CD14 MicroBeads (Miltenyi Biotec Inc.) and cultivated for 5 days in serum-free X-VIVO 15 medium (Lonza Group Ltd) with 4000 U/ml Granulocyte-macrophage colony-stimulating factor (GM-CSF) and 1250 U/ml interleukin (IL)-4 (PeproTech) to obtain immature DCs (iDCs). The iDCs were cultured for another 24 h with an IL-Mix containing 10 ng/ml IL-1β, 10 ng/ml IL-6 and 10 ng/ml tumor necrosis factor alpha (Miltenyi Biotec Inc.) and 15 U/ml of the different FVIII variants, leading to mature DCs (mDCs).

In parallel $CD4^+CD25^-$ T cells from the same donor were separated from regulatory T cells using the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotec Inc.). After the purification, the T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE; BioLegend)[14] and cultured for 2 days in X-VIVO 15 medium with 20 U/ml IL-2 (Miltenyi Biotec Inc.).

The T cells were harvested for co-cultivation and added to the mDCs in a ratio of DCs:T cells of about 1:10. The co-cultivation was performed in fresh X-VIVO 15 medium for 9 days.

On day 9 the T cells were harvested and labeled, in addition to the already performed CFSE-labeling, with 7-AAD (BioLegend), CD25 APC, and CD4 VioBlue (Miltenyi Biotec Inc.) for flow cytometric analyses. Flow cytometry was performed using a FACSVerse (Becton Dickinson GmbH). The cells were gated on viable $CD4^+$ T cells. This population was further analyzed for proliferation based on the CFSE signal (gating strategy see Figure S1 in supporting information. The assay was performed once per donor, due to the large number of cells needed for one analysis.

2.6 | Statistical analyses

All statistical analyses were performed using the software Prism (GraphPad Software). The Wilcoxon test was used to compare two groups. Significant results are marked with asterisks, depending on the significance. One asterisk corresponds to P-value ≤ .05.

3 | RESULTS AND DISCUSSION

3.1 | Deimmunization of FVIII

The EpiMatrix and ClustiMer tools were used to identify which peptides of the FVIII sequence were most likely to be bound by MHC class II. The analyzed FVIII sequence comprised 1514 amino acids, excluding the signal sequence and 818 amino acids of the B domain. *In silico* analysis revealed 52 potentially immunogenic peptide clusters. Of these clusters, 36 clusters overlapped with already published, experimentally determined peptides that bound to MHC class II.[15–20] For 12 of the 52 clusters no amino acid substitutions could be recommended, either due to potential interference with

regions important for activity, binding, or stability, or due to the lack of possible modifications that would reduce binding to the MHC class II. To deimmunize the remaining 40 clusters, 74 substitutions were recommended. For some clusters up to three possible substitutions were considered. In the case that an additional substitution only led to a slight reduction in the cluster immunogenicity score, this substitution was set aside. Additionally, substitutions in five clusters were completely set aside, as the total score of the cluster was already low and the predicted improvement by the substitutions was marginal. These exclusion criteria led to the reduction of the number of potential substitutions from 74 to 57 substitutions that could be incorporated into the FVIII.

Modification of the FVIII sequence was performed in consecutive rounds and the most important readout was the activity of the variants (Figure 1). Each of the resulting variants was tested in two separate transfections. To compare results from different experiments the activity of the modified FVIII variants was normalized to the activity of the unmodified FVIII from the same experiment, leading to a relative activity for each variant. The relative activity had to be at least 50% to transfer a substitution or a combination thereof to the next round.

Of the 57 single substitutions, 38 were successfully incorporated as single substitutions. To keep the number of combinations reasonable, only one substitution per immunogenic cluster was transferred to the next round. Hence, any substitution that resulted in lower relative FVIII activity was excluded. This led to the selection of 25 substitutions, which were transferred to the second round of screening.

Next, several substitutions were combined in different domains of the FVIII sequence. Again, variants leading to relative activities above 50% were taken to the next round. For relative activities below 50% the interfering substitutions were determined by the expression of various combinations of the substitutions based on a design of experiment (DOE) matrix. This was performed for two sections: One comprising the A2 domain and the other comprising most of the A3, C1 and C2 domain. The remaining substitutions in the A2 section were modelled in a half-factorial design, whereas the remaining substitutions in the A3C1C2 section were analyzed in an eighth fraction fractional design. This led to an exclusion of one substitution from the A2 section and five substitutions from the A3C1C2 section, leading to combinations with relative activities above 50%.

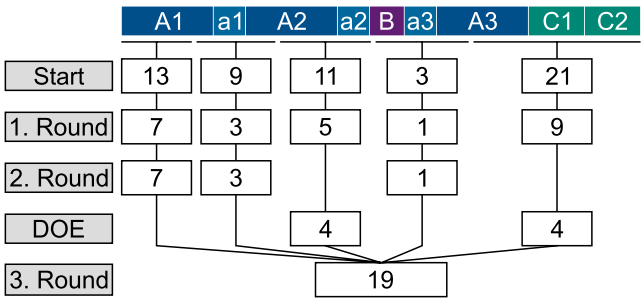

FIGURE 1 Schema for the incorporation of amino acid substitutions into the factor VIII (FVIII) sequence. The numbers indicate the substitutions leading to active FVIII variants in the respective rounds. The substitutions were structured in clusters. In the first round the substitutions were tested individually, whereas combinations of the most successful substitutions were tested in the second round, during design of experiment testing and in the third and final round

In the last round all remaining 19 substitutions were combined in one molecule. This variant revealed a relative activity of 106%. Substitutions that were incorporated in the final version are N79S, S112T, L160S, L171Q, V184A, N233D, I265T, N299D, Y426H, S507E, F555H, N616E, L706N, Y748S, K1837E, N2038D, S2077G, S2315T, and V2333A. Of the initial 57 substitutions, 19 substitutions were successfully incorporated into the FVIII sequence, leading to a new variant to be called "FVIII-19M."

Although 19 substitutions were incorporated, the results revealed that some clusters could not be altered, neither *in silico* nor experimentally. Some substitutions could not be incorporated although they were not predicted to interfere with functional sites or were not known to be associated with hemophilia A. It is most likely that these substitutions had a negative influence on the structure of FVIII.

Comparing the distribution of predicted immunogenic clusters no large differences were detectable between the domains. However, for substitutions that could be incorporated, it became obvious that most of the 19 substitutions were located in the A1 and A2 domain. This might be due to the importance of the A3, C1, and C2 domain for the functionality of the activated FVIII in the tenase complex.

The final FVIII-19M was calculated to have a predicted immunogenicity score of −10.55 compared to the initial score of 7.01 for the unmodified FVIII, a reduction of 17.56 points on the normalized scale (Figure 2).

The negative immunogenicity score reflects a lower density of potentially immunogenic epitopes compared to a set of random proteins of the same length.[10] The score for FVIII-19M is also lower than the median score for the entire human proteome, which is −9.05.[21] This suggests that FVIII-19M would be less immunogenic in hemophilia A patients and less likely to activate the immune system. Taking into consideration that this reduction was achieved by changing only 1.25% of the FVIII amino acid sequence, the process of deimmunization was very efficient.

3.2 | Characterization of the partially deimmunized FVIII

The activity and antigen values of the FVIII-19M were compared to the values of the unmodified FVIII. This was done for four different production batches. For each batch the relation of FVIII activity to the amount of FVIII antigen, the specific activity in %, was calculated. The results are illustrated in Figure 3. The analysis revealed similar results with no significant difference between the medians, comparing the four batches of unmodified FVIII and FVIII-19M. Additionally, the results are in line with published specific activities for moroctocog alfa.[22]

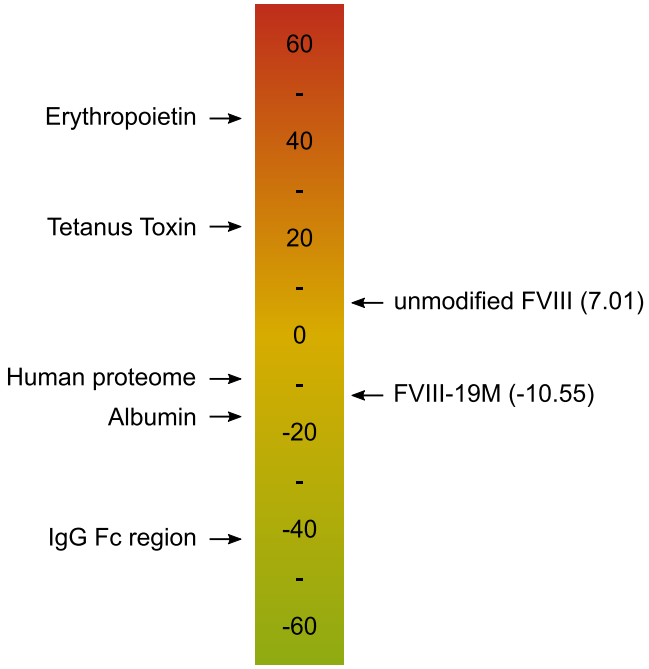

FIGURE 2 Immunogenicity scale. The scores indicate the immunogenicity of a protein calculated by the EpiMatrix system, representing >98% of the human population. The immunogenicity score for each protein is normalized to the median score that was determined for 100,000 random proteins of 1000 amino acids in length. This normalization makes it possible to compare proteins of different lengths. As shown, the score of FVIII-19M has a lower epitope density than the median random protein score, and its score is lower than the median score for the entire human proteome

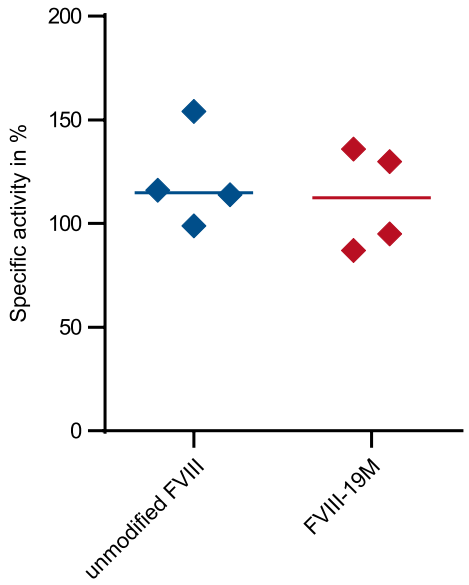

FIGURE 3 Specific activities of four productions of unmodified factor VIII (FVIII) and FVIII-19M. The specific activity in % is calculated as the relation of chromogenic FVIII activity to amount of antigen. The line indicates the median of the four measurements

The clotting time for the different FVIII products was determined using the ROTEM method. By adding different concentrations of FVIII to the test, an increase of the clotting time was detected in correlation to decreasing concentrations of FVIII (Figure 4).

Determining the clotting time for FVIII-19M compared to the unmodified FVIII, moroctocog alfa, and simoctocog alfa, a slightly prolonged clotting time was detected for FVIII-19M. However, all clotting times only varied between 120 and 160 s at 1 U/ml FVIII, which was still in the normal clotting time range of 100 to 240 s in healthy people.[23]

A western blot, detecting light chain and heavy chain of FVIII, revealed that FVIII-19M and the unmodified FVIII were secreted nearly in equal parts as double-chain FVIII, comprising heavy (red band) and light chain (green bands), and as single-chain FVIII (yellow band). In contrast to that, moroctocog alfa and simoctocog alfa were mainly present in the double-chain pattern (Figure 5).

As neither the B domain deletion nor the incorporated substitutions interfered with the furin cleavage site responsible for the generation of the double-chain variant, it is unlikely that the high amount of single chain was due to variations in the amino acid sequence. The strongest influence might have been the production cell line. Different expression levels of furin or other intracellular proteases in the cell lines HEK, CHO, and CAP-T, might have led to different cleavage patterns of the presented FVIII products. Additionally, the more extensive purification process for simoctocog alfa and moroctocog alfa might have been an influence. However, this issue was not evaluated further, as the single-chain variant was not expected to have a negative influence on the functionality or immunogenicity of FVIII. Notably, a rFVIII product especially designed to be secreted as a single chain is already approved as a therapeutic FVIII product.[24,25]

The different molecular weights of the heavy chains were due to the deletion of a larger B domain fraction in moroctocog alfa and simoctocog alfa. The slight differences in the molecular weight of the light chain were probably due to differences in processing and post-translational modifications,[26] mainly glycosylations,[22,27] again due to the different production cell lines.

Although the FVIII-19M clotting activity was confirmed by functional analyses, the activation of FVIII-19M by thrombin was also evaluated on a western blot. This revealed that FVIII-19M, the unmodified FVIII, and moroctocog alfa were spliced by thrombin and the characteristic fragments for A1 domain, A2 domain, and light chain were detectable (Figure 6).[26]

Two fragments were additionally detected for FVIII-19M and the unmodified FVIII. The fragment detected at around 20 kDa consists of the remaining B and a3 domain of the single-chain variant. The single chain was cleaved at all three thrombin cleavage sites but due to missing furin cleavage, the combination of B domain and a3, too small to be detected individually, was still visible on the blot. The fragment of about 90 kDa represents the A1 and A2 domain and was derived either from incomplete activation of the heavy chain, only cleaved at R759, or of the single chain, only cleaved at R759 and R1708. In general, the thrombin activation showed the expected pattern of FVIII fragments, confirming that neither the insertion of

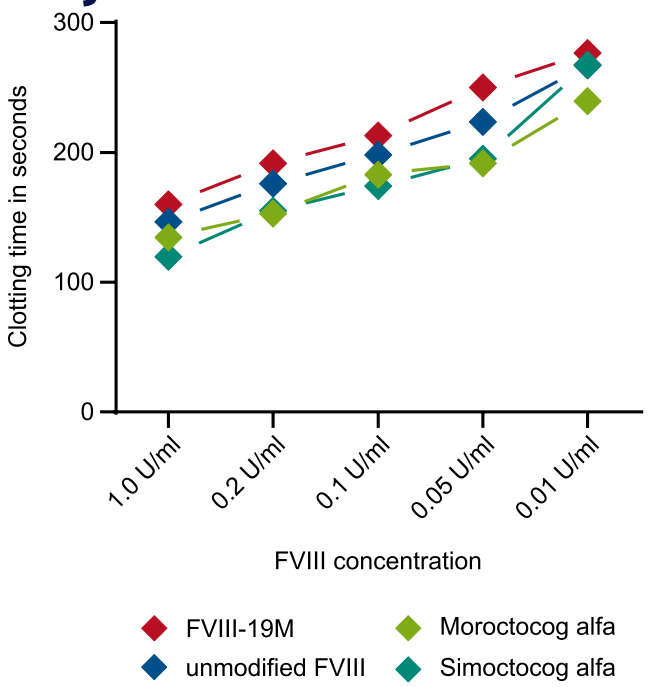

FIGURE 4 Clotting time for FVIII-19M, unmodified factor VIII (FVIII), moroctocog alfa, and simoctocog alfa. Different FVIII concentrations were analyzed. The measurements were performed in duplicate and the mean values are displayed. Standard deviation of each duplicate was below 6 s

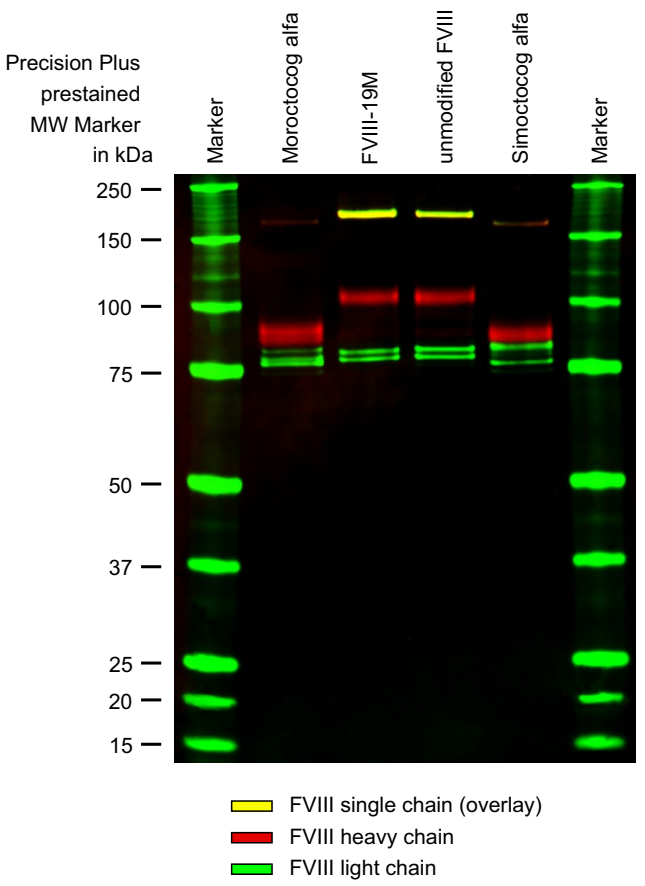

FIGURE 5 Western blot specifically detecting the factor VIII (FVIII) heavy and light chain. The different FVIII products were applied at a concentration of 5 U/ml. The heavy chains are indicated in red and the light chains are indicated in green. Single-chain FVIII, consisting of heavy and light chain, is indicated in yellow, due to the overlay of green and red. MW, molecular weight

the substitutions nor the single-chain conformation impaired the thrombin cleavage.

3.3 | In vitro evidence for the deimmunization

To determine whether $CD4^+$ T cells were less likely to be activated by FVIII-19M, due to reduced presentation of FVIII-19M peptides on the surface of DCs, an *in vitro* DC--T cell assay was established. The assay was performed with cells from healthy donors. It is known that healthy people can possess non-inhibitory anti-FVIII antibodies[28,29] and FVIII-reactive $CD4^+$ T cells, which are often not depleted during ontogeny.[30–32] The assay set-up comprised DCs and $CD4^+CD25^-$ T cells. The depletion of $CD4^+CD25^+$ regulatory T cells was crucial, so that the activation of FVIII-specific $CD4^+$ T cells could be detected.[33] After 9 days of co-cultivation, the proliferation of viable $CD4^+$ T cells was assessed by flow cytometry (see Table S1 in supporting information). In total, 23 different healthy donors were analyzed. Those donors that were observed to have a higher $CD4^+$ T cell proliferation response to DCs that were only treated with the IL-Mix compared to DCs treated with the IL-Mix and moroctocog alfa or unmodified FVIII were excluded from the final analysis. These healthy donors were assumed not to react to FVIII, which can be expected, as not all healthy donors possess $CD4^+$ T cells against FVIII.[34] The results of the remaining 17 healthy donors were included in the final statistical analysis. Figure 7 displays the difference between the $CD4^+$ T cell proliferation to DCs stimulated with IL-Mix plus FVIII-19M and to DCs stimulated with IL-Mix plus unmodified FVIII. Results below 0 indicate a reduced T cell response to FVIII-19M compared to unmodified FVIII. A reduction in proliferation was detected in most donors. However, results above 0 were detected in five donors. The increase in proliferation was below 10% for these donors, whereas much more dramatic decreases in proliferation were detected in the group of donors that demonstrated a reduced response to FVIII-19M. Overall, the data revealed a significant reduction in $CD4^+$ T cell proliferation to DCs stimulated with IL-Mix and FVIII-19M.

For six of the donors included in the proliferation analysis, the HLA-DR genotype was available. This was used to calculate an iTEM score[13] for the FVIII-19M and the unmodified FVIII for each donor, based on their specific HLA-DR genotype. For each donor the difference in the iTEM scores between FVIII-19M and unmodified FVIII was plotted against the corresponding difference in $CD4^+$ T cell proliferation (Figure 8).

iTEM analysis for Donor #2 (HLA-DRB1*13/HLA-DRB1*08), #8 (HLA-DRB1*01/HLA-DRB1*04), and #10 (HLA-DRB1*01/HLA-DRB1*11) revealed large decreases in the iTEM score for FVIII-19M compared to the unmodified FVIII, suggesting that the

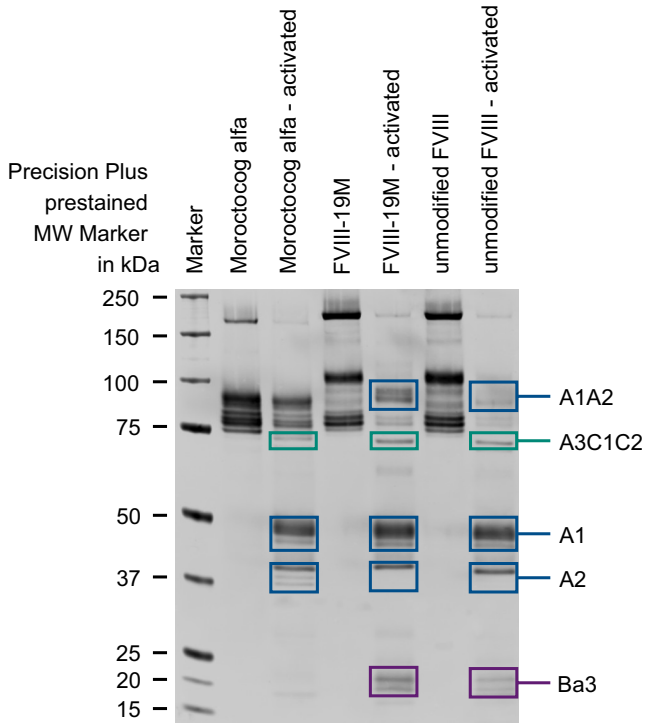

FIGURE 6 Factor VIII (FVIII) products activated by thrombin. Each product was applied in its non-activated and activated form. In the non-activated form, the typical bands for the single chain, heavy chain, and light chain were detectable. After thrombin cleavage additional bands for A1, A2, A1A2, Ba3, and A3C1C2, were detectable. MW, molecular weight

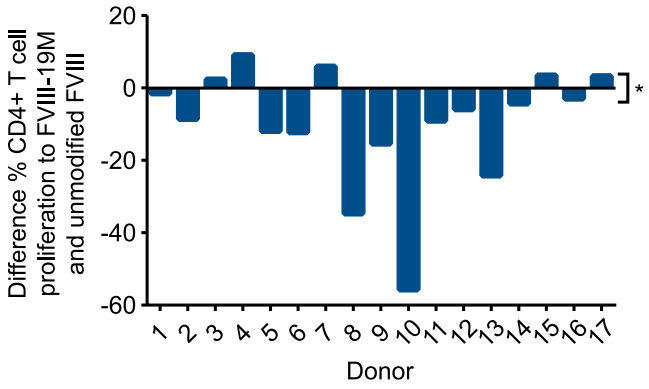

FIGURE 7 Difference between the $CD4^+$ T cell proliferation response to dendritic cells (DCs) stimulated with IL-Mix plus FVIII-19M and DCs stimulated with IL-Mix plus unmodified factor VIII (FVIII). The bars below 0 indicate a reduced $CD4^+$ T cell response to FVIII-19M. The lower $CD4^+$ T cell response to FVIII-19M compared to unmodified FVIII is significant using the Wilcoxon test ($P = .027$)

deimmunization was effective for this combination of alleles, and a corresponding large decrease in proliferation was observed in these three donors. Furthermore, Donor #3 (HLA-DRB1*03/HLA-DRB1*13), who demonstrated an increase in $CD4^+$ T cell proliferation

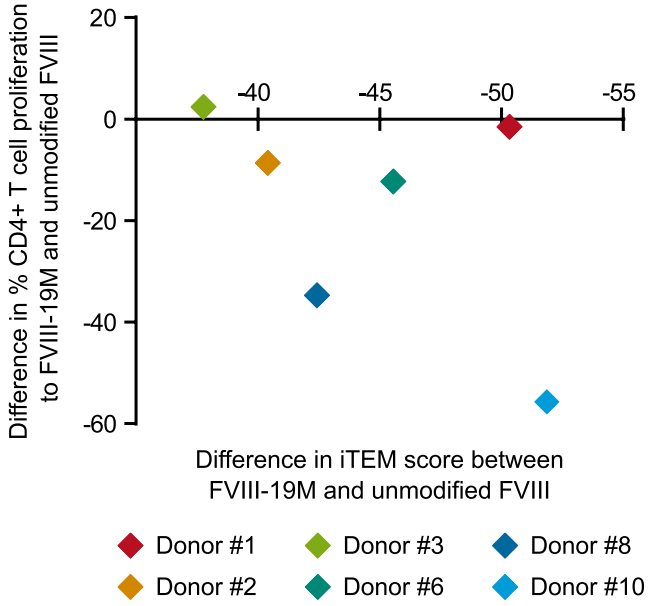

FIGURE 8 Relation between the differences in individual T cell epitope measure (iTEM) scores and proliferation. The iTEM scores for FVIII-19M and unmodified factor VIII (FVIII) were calculated for Donor #1, #2, #3, #6, #8, and #10, based on their human leukocyte antigen (HLA)-DR genotype. The differences of the iTEM scores were plotted against the differences in $CD4^+$ T cell proliferation, determined in the dendritic cell (DC)--T cell assay

to FVIII-19M compared to unmodified FVIII, was the subject that had the lowest difference in iTEM scores, indicating that the combination of 19 substitutions had no impact on the immunogenicity of FVIII for this subject. In contrast, Donor #1 (HLA-DRB1*04/HLA-DRB1*14) and #6 (HLA-DRB1*07/HLA-DRB1*08) did not show as great a decrease in proliferation as would have been expected based on the change in their iTEM scores for FVIII-19M. This shows that immunogenicity is not only dependent on the sequence of the peptides but may also depend on whether the epitopes are processed and presented, based on individual expression of proteases cleaving the protein, as well as on the available $CD4^+$ T cell pool of a donor.[15] Overall, these data revealed that individual *in silico* analyses were consistent with results observed in the *in vitro* DC-T cell assay.

An additional *in silico* analysis, calculating the iTEM scores for the unmodified FVIII and FVIII-19M for each HLA-DR haplotype combination is provided in Table S2 in supporting information. These data indicate that reduced immunogenicity against FVIII-19M can be expected for all genotypes. However, the highest change in iTEM scores was predicted for haplotypes HLA-DRB1*11 and the inhibitor-associated HLA-DRB1*15.

4 | CONCLUSION

The data revealed that *in silico* analysis can identify amino acid substitutions that could be incorporated into FVIII. Surprisingly, despite the large number of substitutions, the modified FVIII-19M remains

functional. However, predicting the consequences of amino acid substitutions for the functionality of FVIII was difficult. This indicates the importance of additional research on the structure of FVIII, to better model the influence of amino acid substitutions on function. Nevertheless, it would be interesting to determine whether the incorporation of further substitutions would still lead to a functional FVIII molecule and whether this would result in a further reduction of the immunogenicity of the molecule.

FVIII-19M can be produced and purified, similar to the unmodified FVIII and different methods comparing the protein structure and functionality revealed only minor differences to unmodified FVIII, moroctocog alfa, and simoctocog alfa. However, further investigations are needed, especially mass spectrometric analyses, to reveal glycosylation and sulfation in detail.

In an *in vitro* DC--T cell assay the incorporation of 19 substitutions into the FVIII sequence led to a reduction in $CD4^+$ T cell proliferation compared to the unmodified FVIII molecule. However, reactivity to FVIII-19M was not completely absent. As several immunogenic clusters of FVIII could not be altered, this result is not surprising. Unfortunately, it was not possible to obtain adequate amounts of blood from hemophilia A patients to perform the DC--T cell assay, which would give even more representative results for what could be expected in a clinical setting.

Whether the incorporated 19 amino acid substitutions also disrupt B cell epitopes was not further investigated. Although it is known that T and B cell epitopes can cluster within a short sequence,[34–36] experimental proof is complicated, due to the fact that the exact epitopes of most anti-FVIII antibodies are unknown.

To gain further proof of the concept and to support the *in vitro* results, a detailed analysis of the peptides presented on the MHC class II is needed using MHC-associated peptide proteomics (MAPPS) or other techniques. As shown by Jankowski et al.,[37] this kind of assay clearly detects FVIII-specific peptides presented on the MHC class II and can clearly distinguish between different variants of FVIII molecules. If this analysis supports the *in vitro* DC-T cell assay results, success *in vivo* with the partially deimmunized FVIII-19M would be likely.

The confirmation of the *in vitro* results *in vivo* was not feasible, due to the lack of an appropriate mouse model. Although a total FVIII-knockout mouse model[38] and a humanized mouse model with human DCs, T, B, and natural killer cells[39–42] are available, the combination is still missing. The current absence of such a mouse model underlines the importance of a reliable *in vitro* assay with human cells for the prediction of FVIII immunogenicity.

Taken together FVIII-19M is the first FVIII molecule that was systematically deimmunized throughout the whole sequence, revealing reduced immunogenicity while retaining its functionality. The data indicate that an *in silico* deimmunization approach can be applied even to a large molecule such as FVIII. The constructed FVIII-19M could improve FVIII therapy, as it might reduce the initial risk of antibody development in previously untreated patients but also in previously treated patients, who can still develop antibodies after years of treatment.[43] Evidence is accumulating that *in silico* models provide a good base for prediction of immunogenicity and should be applied during the development of recombinant proteins. For example, a *post hoc* analysis of the phase III trial for vatreptacog alfa clearly demonstrated that where amino acid substitutions were introduced without preclinical computational analysis, anti-drug antibodies resulted during the clinical trial due to a immunogenic neo-epitope.[44]

With the advancing technological knowledge in this field and accompanied by adequate biological assays the development of less immunogenic natural proteins for substitution therapy is within reach.[45] FVIII-19M therefore represents a new generation of less immunogenic FVIII molecules and it would be intriguing to establish further proof of concept in a clinical setting.

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CONFLICTS OF INTEREST

K. Winterling, J. Daufenbach, S. Kistner, and J. Schüttrumpf are employees of Biotest AG. A. S. De Groot is a senior officer and shareholder of EpiVax, Inc., a privately owned biotechnology located in Providence, RI. W. D. Martin is a senior officer and shareholder of EpiVax, Inc., a privately owned biotechnology located in Providence, RI. W. D. Martin and A. S. De Groot acknowledge that there is a potential conflict of interest related to their relationship with EpiVax and affirm that the information represented in this paper is original and based on unbiased observations. K. Winterling, W. D. Martin, A. S. De Groot, J. Daufenbach, and S. Kistner are inventors on deimmunized factor VIII–related patents. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter.

AUTHOR CONTRIBUTIONS

K. Winterling designed, performed, and interpreted the research, and wrote the manuscript. W. D. Martin performed the *in silico* analyses. A. S. De Groot, J. Daufenbach, and S. Kistner assisted with the design of the experiments and with interpreting the data. J. Schüttrumpf, W. D. Martin, A. S. De Groot, and S. Kistner critically reviewed the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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