Minimal Essential Human Factor VIII Alterations Enhance Secretion and Gene Therapy Efficiency

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One important limitation for achieving therapeutic expression of human factor VIII (FVIII) in hemophilia A gene therapy is inefficient secretion of the FVIII protein. Substitution of five amino acids in the A1 domain of human FVIII with the corresponding porcine FVIII residues generated a secretion-enhanced human FVIII variant termed B-domain-deleted (BDD)-FVIII-X5 that resulted in 8-fold higher FVIII activity levels in the supernatant of an in vitro cell-based assay system than seen with unmodified human BDD-FVIII. Analysis of purified recombinant BDD-FVIII-X5 and BDD-FVIII revealed similar specific activities for both proteins, indicating that the effect of the X5 alteration is confined to increased FVIII secretion. Intravenous delivery in FVIII-deficient mice of liver-targeted adeno-associated virus (AAV) vectors designed to express BDD-FVIII-X5 or BDD-FVIII achieved substantially higher plasma FVIII activity levels for BDD-FVIII-X5, even when highly efficient codon-optimized F8 nucleotide sequences were employed. A comprehensive immunogenicity assessment using in vitro stimulation assays and various in vivo preclinical models of hemophilia A demonstrated that the BDD-FVIII-X3 variant does not exhibit an increased immunogenicity risk compared to BDD-FVIII. In conclusion, BDD-FVIII-X5 is an effective FVIII variant molecule that can be further developed for use in gene- and protein-based therapeutics for patients with hemophilia A.

INTRODUCTION

Hemophilia A (HA) is an X-linked, recessive bleeding disorder caused by deleterious mutations in the F8 gene.1–6 Current treatment for HA predominantly relies on human FVIII (hFVIII) protein replacement therapy (PRT) administered through relatively frequent intravenous infusions and is, in a subgroup of patients with severe HA, complicated by the development of inhibitory antibodies against the exogenously delivered FVIII.6,7 Gene therapy, on the other hand, offers the prospect of a functional cure with a single therapeutic dose and might have, in addition to providing substantial and constantly present FVIII levels, a tolerizing effect, which might reduce the risk of inhibitor development.8–12

The results from gene therapy clinical trials using adeno-associated virus (AAV) to treat hemophilia B are encouraging,13,14 and the recent release of preliminary data for an AAV5-FVIII human clinical trial demonstrates sustained FVIII activity with no significant adverse events reported.6,15,16 More recently however, three years of follow-up data also identified a decline in FVIII expression levels over time, suggesting that this therapy may not last lifelong17 and causing the US Food and Drug Administration (FDA) to ask for two years of data from the phase 3 trial to show substantial evidence of a durable effect.18

HA gene therapy using the AAV vector system is complicated by three key issues. One challenge is the size of the BDD-F8 cDNA, which is around 4.4 kb in length, bringing the final vector size close to the canonical 4.7 kb AAV genome. Small and efficient promoters are therefore essential for packaging and delivering FVIII using AAV vectors, since little room is left for the addition of genetic elements such as promoters and enhancers to increase transgenic expression.
The second limitation is inefficient secretion of FVIII, likely caused by a slow folding process of the factor in the endoplasmic reticulum. Third, upon ectopic overexpression of FVIII, an unfolded protein stress response may be triggered, the consequences of which are not fully understood.

Efforts to increase therapeutic FVIII activity levels include codon optimization of the F8 gene, engineering FVIII to have a higher specific activity, and modulating the FVIII secretory pathway by influencing the interactions between FVIII and immunoglobin binding protein (BiP), calnexin, and calreticulin. Addition of non-human sequences may foster protein expression levels but at the same time might increase the potential risk for unwanted anti-drug antibody formation.

Porcine FVIII (pFVIII), which shares approximately 65% sequence identity with hFVIII (excluding the B-domain), was shown in transfection studies and by preclinical ex vivo gene therapy approaches to be expressed 10- to 100-fold more efficiently than hFVIII. This effect could be narrowed down by demonstrating that a porcine/human BDD-FVIII hybrid construct termed ET3i containing the porcine A1 and A3 domains retained the higher expression levels of pFVIII in various expression systems.

We therefore hypothesized that a subset of amino acids in pFVIII, and likely in domains A1 and/or A3, may facilitate its enhanced secretion. By implementing a systematic screening strategy, five key amino acids in pFVIII were identified (X5), which, when introduced into the backbone of hBDD-FVIII, resulted in a highly efficiently secreted protein. Our study demonstrated that this protein, called BDD-FVIII-X5, could be the basis for a promising gene therapy strategy in which an effective FVIII variant with enhanced secretion properties may help to substantially reduce the vector dose for treating HA patients.

RESULTS

Identification of the Secretion-Enhancing Amino Acid Residues in the HC of pFVIII

Initial testing in vitro demonstrated that replacement of the human FVIII heavy chain (hHC) with porcine heavy chain (pHC) resulted in enhanced secretion of BDD-FVIII (data not shown), in line with previous reports. However, swapping of hHC with pHC would result in a large number of amino acid substitutions with potentially negative immunological consequences. We therefore designed a strategy to narrow down the region within pHC that is responsible for enhanced hFVIII secretion. Because the isolated hHC is particularly inefficiently secreted as compared to full-length FVIII or BDD-FVIII, we elected to use hHC as the template molecule for the screening assays. A full set of hHC variants containing corresponding regions of pFVIII were generated in the A1 and A2 domains (Figure 1A). In vitro testing using dual chain delivery of the human light chain (hLC) with the hHC variants showed that of the 13 FVIII-HC constructs tested, those containing porcine amino acids 82-176 (construct xHC p82-176) exhibited the highest procoagulant activity (Figure 1B). Similar substitutions in human BDD-FVIII confirmed this observation (Figure 1C). An amino acid sequence alignment revealed that within amino acids 82-176 there are 10 amino acids that differ between pFVIII and hFVIII: I86V, Y105F, A108S, D115E, Q117H, F129L, G132K, H134Q, M147T, and L152P (Figure S1). Therefore, we designated xHC p82-176 as HC-X10 in the following experiments.

We next determined if all 10 porcine amino acids are required to enhance hFVIII secretion by adopting a negative selection strategy.
In this strategy, each of the 10 porcine amino acids in the HC-X10 hybrid molecule was replaced with its human counterpart, with the assumption that a significant change would reduce the efficiency of HC-X10 secretion. The negative selection results identified five porcine-to-human amino acid exchanges (V86I, S108A, K132G, T147M, and P152L) that reduced HC-X10 performance by at least 20% (Figure S2), suggesting that these 5 amino acids may play a key role in facilitating FVIII secretion. This hypothesis was tested by constructing HC-X5, a hHC containing these 5 porcine amino acids.

Testing this construct in vitro revealed that delivery of either HC-X5 or HC-X10 in conjunction with hLC resulted in an approximately 10-fold increase in FVIII activity (Figure 2A). While both HC-X5 and HC-X10 had significantly higher FVIII activity compared to hHC, they were not significantly different from each other, indicating that the 5 positions selected are indeed the key porcine amino acid residues facilitating FVIII secretion. We next evaluated whether the observed effect is also seen in the context of a human BDD-FVIII molecule that had been engineered to contain the X5 alteration. Testing in vitro revealed an 8-fold increase in FVIII clotting activity compared to BDD-FVIII (Figure 2B). Taken together, these results provided evidence that the X5 amino acid alteration had transferred improved secretion properties of pFVIII to hFVIII, as it was able to enhance the levels of secreted hFVIII activity in vitro.

A pulse-chase experiment was used to verify that the increased FVIII activity measured in the medium is due to the increased secretion of BDD-FVIII-X5. Following labeling of the newly translated proteins with 35S-methionine and 35S-cysteine, the secreted BDD-FVIII variants were immunoprecipitated using a FVIII-specific antibody. More BDD-FVIII-X5 was indeed detected in the medium compared to BDD-FVIII (Figures S3A and S3B), indicating that the X5 alteration led to more efficient FVIII secretion.

The results so far indicated that the X5 variant improves secretion of hFVIII but left open the possibility that it also affects FVIII’s cofactor activity, causing an increased specific activity of the X5 variant. We therefore produced recombinant BDD-FVIII and the X5 variant thereof using a Chinese hamster ovary (CHO)-production cell line. A silver gel of the purified material revealed for both proteins the expected pattern of distinct bands for the HC and the LC, as well as a tiny band of single-chain BDD-FVIII, caused by an incomplete intracellular processing in CHO cells (Figure 3A). Whether the increased intensity of this band for BDD-FVIII-X5 points toward a fraction of the protein being secreted as single chain remains to be addressed in further studies. Importantly, the specific activity of the X5 variant was similar to that of BDD-FVIII (Figure 3B), indicating that the biochemical and biophysical functionality of BDD-FVIII-X5 is not compromised and that the sole effect of the X5 alteration is to enable efficient secretion of BDD-FVIII.

Delivery of AAV Carrying BDD-FVIII-X5 Produced High Levels of FVIII in a HA Murine Model
To demonstrate suitability of BDD-FVIII-X5 for AAV delivery, we generated an AAV8-BDD-FVIII-X5 vector in which the BDD-F8 gene was under the control of a minimal mouse liver-specific transthyretin (TTR) promoter/enhancer assembly39 (Figure 4A). AAV8-BDD-FVIII-X5 was administered to immunocompetent HA mice at a high dose (4 × 1012 vector genomes [vg]/mouse, ~1.6 × 1013 vg/kg) and a low dose (1 × 1011 vg/mouse, ~4 × 1012 vg/kg), and blood plasma samples were collected over the course of 22 weeks.

The FVIII expression as measured by a one-stage coagulation assay (activated partial thromboplastin time [aPTT]) reached its peak level at week 4 post-injection for the high-dose group (Figure 4B), and at 6 weeks post-injection for the low-dose group (Figure 4C). At high dosage, mice receiving AAV8-BDD-FVIII expressed around 0.4 U/mL FVIII, whereas mice receiving AAV8-BDD-FVIII-X5 expressed ~10–12 U/mL FVIII (Figure 4B). At the low dose, the peak level of FVIII expression was 0.2 U/mL FVIII, and that of AAV8-BDD-FVIII-X5 was over 2 U/mL FVIII (Figure 4C). Over the 22 weeks
tracked, mice receiving the AAV8-BDD-FVIII-X5 vector consistently exhibited a high level of FVIII activity.

The X5 Alteration Confers Also Improved Secretion when Introduced into Codon-Optimized BDD-FVIII Nucleotide Sequences

Since the wild-type nucleotide sequence of human F8 is poorly expressed, current HA gene therapy vectors critically depend on codon-optimized sequences to increase the expression of FVIII to therapeutically relevant levels. It was therefore important to test whether the observed benefit of the X5 alteration would be diminished or even lost when introduced to a codon-optimized sequence background. We chose three codon-optimized BDD-F8 sequences, all of which give rise to improved expression compared to the wild-type nucleotide sequence but to a different extent. Specifically, CO1 codon optimization was based on the nucleotide sequence published by Ward et al.,28 CO2 was a nucleotide sequence designed using the Web-based GeneArt algorithm, and CO3 was designed in Takeda. The three nucleotide sequences and the corresponding X5 variants were cloned into a liver-specific expression cassette and AAV8-based vectors were generated. Vectors were then administered to FVIII knockout (KO) mice at a dose of $4 \times 10^{11}$ vg/kg body weight (corresponding to approximately $1 \times 10^{11}$ vg/mouse) and plasma samples collected for up to 8 weeks. Resulting FVIII activity levels were consistently higher for the respective X5 variants at all time points measured (Figure 5). There was also no trend seen toward a diminishing improvement of the X5 alteration with increasing expression caused by the codon-optimized sequences; even with sequence CO3, showing a 25-fold higher expression than the non-codon-optimized wild-type sequence, X5 gave rise to a 7-fold increase in FVIII plasma levels.

To exclude that the observed difference in FVIII levels was due to preferential transduction by the X5 variants, we analyzed the livers of representative animals treated with CO3 and CO3-X5, the pair of vectors causing the highest FVIII expression levels, for vector copy numbers (VCNs) and $F8$ mRNA levels (i.e., transduction efficiency). Similar VCNs and mRNA levels were obtained for the two groups (Table S1), indicating that the difference in measured FVIII activity levels in plasma was indeed due to a more efficient secretion of the BDD-FVIII-X5 variant.

To confirm the biologic potency of BDD-FVIII-X5, a mouse tail-tip bleeding assay was performed. Administration of CO3-X5 to FVIII KO mice resulted in a trend for reduced blood loss over 60 min for the lowest dose tested ($4.5 \times 10^{11}$ vg/kg) compared to the buffer-

![Figure 3. The X5 Alteration Does Not Affect the Specific Activity of BDD-FVIII](image)

(A and B) Recombinant BDD-FVIII and BDD-FVIII-X5 were produced in CHO cells and purified to apparent homogeneity. The X5 alteration gave rise to a comparable pattern of protein fragments (A) and showed a specific activity that was similar to that of BDD-FVIII (B). Note that the image of the silver gel was processed to only show relevant lanes. Fl rFVIII, full-length recombinant FVIII (Advate).

![Figure 4. In Vivo Performance of AAV8-Vectorized BDD-FVIII-X5 Using the Human Wild Type (WT) FVIII Nucleotide Sequence](image)

(A) Schematic representation of the vector design of AAV8-BDD-FVIII and AAV8-BDD-FVIII-X5. (B and C) Vectors were injected into hemophilia A (HA) mice (C57BL/6/SvJ29S) at (B) a high dose of $4 \times 10^{11}$ vg/mouse ($n = 4$) and (C) a low dose of $1 \times 10^{11}$ vg/mouse ($n = 5$). The FVIII activity levels in plasma were determined by a 1-stage coagulation assay. ITR, inverted terminal repeats; TTR, transthyretin promoter; pA, PolyA tail; vg, vector genomes.
We further assessed the protein’s immunogenicity risk by using a human PBMC-based in vitro stimulation assay, which allows the detection of potential anti-BDD-FVIII-X5-specific T cell responses with high sensitivity (Figure 6D). Dendritic cells from 20 representative donors were loaded with the respective peptide pools and co-cultured with autologous CD4\(^+\) T cells for six days to allow expansion of potential BDD-FVIII- or BDD-FVIII-X5-specific T cells. After co-culture, T cells were re-stimulated with fresh peptide-loaded antigen-presenting cells from the same donors for 18 h and secreted interferon \(\gamma\) (IFN\(\gamma\)) levels measured as marker for T cell activation. Significant differences in IFN\(\gamma\) levels between BDD-FVIII and BDD-FVIII-X5 were not observed, indicating that the BDD-FVIII-X5-specific peptide sequences did not provoke an increase in T cell activation (Figure 6E). Together, these preclinical data support the conclusion that the X5 alteration does not pose an additional immunogenic risk to BDD-FVIII.

### No Evidence for X5-Specific Inhibitory Antibody Formation in HA Rats

To further evaluate whether inhibitory antibodies could potentially develop against X5-specific sequences in BDD-FVIII-X5, we designed an experiment as outlined in Figure 7A. FVIII inhibitors were raised in two WAG/Rij\(\bar{y}\)c\(\bar{b}\) HA rats by administration of an AAV8 vector designed to express human BDD-FVIII-X5. The resulting rat plasma, which had a Bethesda titer of 60–110 BU/mL, was depleted of inhibitory antibodies against human wild-type FVIII by biotinylated FVIII, mock-treated with biotinylated BSA, or left untreated. When a BDD-FVIII-X5 concentrate was incubated with these three plasma samples as well as reference control plasma from non-immunized rats, no inhibition of FVIII activity was seen for the plasma that had been pre-treated with biotinylated FVIII, whereas non-treated plasma and plasma treated with biotinylated BSA caused a reduction in FVIII activity (Figure 7B). This result was interpreted to mean that detectable inhibitors specifically directed against sequences containing the 5 porcine amino acids of X5 did not develop in the WAG/Rij\(\bar{y}\)c\(\bar{b}\) HA rats, as these would have remained in the supernatant of the FVIIIdpleted plasma and been effective against BDD-FVIII-X5.

#### DISCUSSION

Inefficient FVIII secretion remains one of the major limitations for HA gene therapy using AAV vector delivery.\(^{42}\) Previous efforts to increase FVIII secretion and expression primarily focused on utilizing codon optimization and reported mechanisms that enhance FVIII secretion.\(^{39,43}\) The well-described fact that pFVIII secretes substantially more efficiently than hFVIII provided a unique opportunity to design new BDD-FVIII variants. The combination of positive and negative screenings allowed us to create BDD-FVIII-X5, which is a human BDD-FVIII that harbors only 5 amino acids from pFVIII-X5, and retains enhanced secretion properties (Figure 2). At the same time, the BDD-FVIII-X5 variant proved fully functional, demonstrated by a purified recombinant version having a similar specific activity as the Refacto-like BDD-FVIII (Figure 3).

Efficient secretion of BDD-FVIII-X5 was evident in in vitro and in vivo systems. In a murine HA model, an AAV vector carrying the BDD-F8-X5 gene resulted in substantially enhanced expression when compared to native BDD-F8. In the initial experiment, the native human F8 coding sequence was used (Figure 4). Nevertheless,
FVIII codon optimization was reported to greatly improve FVIII expression, and in fact all vectors currently being used in clinical HA gene therapy trials rely on codon-optimized F8 sequences. Since F8 codon optimization is expected to improve transcription/translation but not necessarily to facilitate FVIII secretion per se, we anticipated that BDD-FVIII-X5 could have an additive effect on codon-optimized BDD-F8. As shown in Figure 5, incorporation of the X\textsubscript{5} alteration into various codon-optimized BDD-F8 sequences indeed improved BDD-FVIII expression further, indicating that X\textsubscript{5}’s positive effect on expression is downstream of protein translation.

Our approach aimed to identify porcine-specific amino acid residues in FVIII’s HC that confer enhanced secretion but left open whether introduction of additional residues out of pFVIII’s LC would further enhance hFVIII levels. Since an ancillary role in secretion was reported for the porcine ap-A3 domains, BDD-FVIII-X5’s secretion efficiency may not reach that of porcine BDD-FVIII. Another limitation of the study is the lack of expression data for BDD-FVIII-X5 in non-human primates, which are thought to be more predictive to the situation in humans. Another bio-engineered FVIII variant that is being tested in gene therapy is FVIII-V3, which has introduced a peptide solution in humans. Another bio-engineered FVIII variant that is being tested in gene therapy is FVIII-V3, which has introduced a peptide

Development of a gene therapy product enabling robust and substantial improvement in efficacy is particularly relevant in light of the observed vector dose-dependent signs of liver inflammation and anti-capsid immune responses. Various clinical hemophilia gene therapy trials using different AAV capsids (AAV2, AAV8, AAVrh10, and LK03) showed such signs of hepatotoxicity already at a dose of $2 \times 10^{12}$ vg/kg. Although the evoked responses can be usually managed quite well by temporal steroid administration (or even prevented when given prophylactically), improved hemophilia gene therapy approaches should render immune suppression dispensable. This may be achieved by the selection of capsids with a strong tropism for human hepatocytes such as AAV3 or NP40, thereby allowing for lower dosing.

In the context of HA, the biological properties of BDD-FVIII-X5 also appear highly valuable in keeping vector-mediated hepatotoxicity under control. Expression of the efficiently secreted BDD-FVIII-X5 variant might in part compensate for the need to express the BDD-F8 transgene at very high levels, which is challenging because of the size restriction imposed on the choice of the promoter due to the size of the BDD-F8 transgene (4.4 kb) being almost as large as the wild-type AAV vector genome. Perhaps even more importantly, BDD-FVIII-X5 might help reduce the potential risk of triggering an anti-FVIII T cell response, cells were re-stimulated with fresh peptide-loaded, T cell (CD3)-depleted PBMCs for 18 h. (E) IFN\textgamma{} secretion of different donors after 18 h of BDD-FVIII and BDD-FVIII-X5 peptide stimulation measured by ELISPOT. The values are represented as stimulation indexes (SIs) relative to the DMSO control. The 15-mer peptide pools used spanned the entire amino acid substitutions of BDD-FVIII-X5 (m\textsubscript{pool1-4}) and the corresponding BDD-FVIII WT sequences (w\textsubscript{pool1-4}). Significant differences in IFN\textgamma{} levels between BDD-FVIII and BDD-FVIII-X5 were not observed.

Figure 6. Comparative Immunogenicity Analysis of BDD-FVIII-X5 and BDD-FVIII

(A–C) In vivo evaluation using three different murine models of HA. FVIII binding antibodies were measured after 8 weekly tail vein injections of 1 µg of BDD-FVIII and BDD-FVIII-X5 (X5) in E17 mice (A), tolerant E17 mice harboring a hFVIII transgene (B), and E17 mice harboring the human HLA DRB1*1501 MHC class II haplotype (C). Groups were considered significantly different for the anti-FVIII antibody response at a p value < 0.05, using the non-parametric Mann-Whitney U test. MC, model control (an immunogenic FVIII control preparation that is capable of mounting a FVIII antibody response in the tolerant E17 mouse model); ns, non-significant. (D and E) PBMC-based in vitro stimulation assay. (D) Flow chart of the assay. CD14 positive monocytes were isolated from 20 donors, loaded with BDD-FVIII or BDD-FVIII-X5-specific peptides, and differentiated into dendritic cells (DCs). In parallel, CD4 cells were isolated from the same donors and co-cultured for six days with the DCs. To boost potential anti-FVIII T cell responses, cells were re-stimulated with fresh peptide-loaded, T cell (CD3)-depleted PBMCs.

We have addressed the potential concern of BDD-FVIII-X5 immunogenicity in a carefully designed comparative study using an in vitro stimulation assay and three mouse models, two of which were specifically developed for preclinical evaluation of FVIII
immunogenicity risks. The transgenic hFVIII mouse model is particularly suitable to study mechanisms causing break of immune tolerance against hFVIII. To date, patients in hemophilia gene therapy clinical studies have been previously treated and are thereby not expected to develop anti-FVIII antibodies to gene therapy products. The existing data from ongoing clinical studies indeed support this assumption. However, there is a potential risk that previously treated patients might experience an immune response against a FVIII variant. As compared to the ET3 porcine-human BDD-FVIII hybrid that contains more than 150 pFVIII amino acids, BDD-FVIII-X5 is a hFVIII harboring only 5 amino acids from pFVIII (I86V, A108S, G132K, M147T, and L152P), all of which are located in the A1 domain. This domain has not been reported as a major tolerance against hFVIII. To date, patients in hemophilia gene therapy clinical studies indeed support this assumption. However, there is a potential risk that previously treated patients might experience an immune response against a FVIII variant. As compared to the ET3 porcine-human BDD-FVIII hybrid that contains more than 150 pFVIII amino acids, BDD-FVIII-X5 is a hFVIII harboring only 5 amino acids from pFVIII (I86V, A108S, G132K, M147T, and L152P), all of which are located in the A1 domain. This domain has not been reported as a major immunogenic domain, even after extensive analysis of human antibodies against human FVIII. In our evaluation, BDD-FVIII-X5 did not induce an increased immunogenic risk in any of the mouse models (Figures 6A–6C). The PBMC re-stimulation data (Figure 6E) further support the conclusion that the X5 alteration may not increase the immunogenic risk in previously treated hemophilia patients. Finally, a complementary assessment in rats suggested that neo-antigenic epitopes are limited by introduction of the five porcine amino acids (Figure 7).

Although we have been comprehensive in our evaluation, the design of our studies did not address whether continuous expression of BDD-FVIII-X5 (or BDD-FVIII for that matter) will lead to an induction of immune tolerance to hFVIII. Also, we have not exposed larger animal models to BDD-FVIII-X5. Their immune system may respond to the X5 alteration differently than that of rodents, possibly mimicking the human situation more closely. However, larger animal models, such as non-human primates, are not transgenic for hFVIII and hence not tolerant even to native hFVIII due to differences in their FVIII sequence. The immune response against BDD-FVIII-X5 in such animals could therefore be altered because of their high propensity to mount an anti-FVIII immune response against hFVIII even without the X5 insertion. We therefore believe that our selected models are valid in terms of predictability of immunogenic outcomes in humans. At the same time, one should avoid over-interpretation of the data given the caveats inherent to all in vitro and in vivo animal models.

Taken together, we conclude that BDD-FVIII-X5 has great potential as a leading candidate for a more efficient and better tolerated gene therapy for patients with HA.

MATERIALS AND METHODS

**FVIII Expression Plasmids and Mutagenesis**

pFVIII was synthesized by GenScript based on Doering et al. BDD-FVIII and FVIII HC and LC have been described previously.29,36 FVIII expression constructs for testing in cultured cells were driven by a Cytomegalovirus enhancer with a human β-actin promoter (CB). All hybrid human and pFVIII variants including desired point mutations were confirmed by DNA sequencing.

For the in vivo expression of BDD-FVIII (B domain replaced by the 14-amino acid SQ linker sequence) and BDD-FVIII-X5, the human wild-type coding sequence and three codon-optimized sequences (CO1, CO2, and CO3) as well as the corresponding BDD-FVIII-X5 sequences (CO1-X5, CO2-X5, and CO3-X5) were synthesized and cloned into an AAV expression cassette containing a liver-specific murine transthyretin promoter/enhancer combination.39

**Tissue Culture and Transfection**

For evaluating FVIII plasmid performance in vitro, transfected baby hamster kidney (BHK) cells or human embryonic kidney (HEK293) cells (American Type Culture Collection, Manassas, VA, USA) were grown for 6–12 h in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and then replaced with Ham’s F-10 Nutrient Mix (GIBCO, Grand Island, NY, USA) medium with 2% heat-inactivated fetal bovine serum. The media were collected 24 h later, and the activity levels of FVIII in the media were measured. All experiments were performed in triplicate.

**AAV Vector Preparation**

Recombinant AAV8 vectors were produced by a triple plasmid co-transfection method as described previously.29,36 AAV vectors were purified by two rounds of cesium chloride gradient ultracentrifugation followed by an extensive buffer exchange against PBS with 5% D-sorbitol. Alternatively, cell pellets of 1.2 L cultures were processed...
using one round of iodixanol gradient centrifugation followed by an anion-exchange polishing step. Quantitation of the vector genome was done by inverted terminal repeat (ITR)-specific qPCR.52

Animal Procedures
Exon 16-disrupted FVIII KO (HA) mice (B6;129S-E8m1Kaz/J) were obtained from Haig Kazazian (University of Pennsylvania), and exon 17-disrupted FVIII KO mice (B6;129S4-E8m2Kaz) were bred at Charles River (Sulzfeld, Germany). Wag/RijYcb HA Rattus norvegicus rats (strain designation: WAG-F8m1Ycb) were obtained from Haig Kazazian (University of Pennsylvania), and HLA-DRB1*1501 E17 hemophilic mice41 were used for comparative immunogenicity studies. Mice received weekly tail vein injections of 1 μg of BDD-FVIII or BDD-FVIII-X5 for a total of 8 weeks. The E17 human transgenic FVIII mouse line was additionally challenged with a modified FVIII protein known to break tolerance.40 Blood was collected by retro-orbital bleeding at week 6 or by cardiac puncture one week after final FVIII administration. Resulting plasma samples were analyzed for anti-FVIII binding antibodies by direct ELISA as described.40 Studies were analyzed by using non-parametric Mann-Whitney U test to compare statistical significance; a p value < 0.05 was considered significant.

Human peripheral blood mononuclear cell (PBMC) re-stimulation assays were performed at Lonza using 20 individual donors representing a global population distribution of major histocompatibility complex (MHC) class II haplotypes (HLA-DRB1) and peptide pools (15-mer) spanning the X5 amino acid substitutions (m_pool) or corresponding wild-type peptide sequences (w_pool). The CEFT (Cytomegalovirus, Epstein-Barr virus, Flu-virus, and Tetanus toxin) peptide pool (0.5 μM) was used as positive control. In brief, autologous CD4 T cells were incubated with peptide-loaded monocyte-derived dendritic cells from the same donor for 6 days. On day 6, preloaded CD3-depleted PBMCs from the same donor were added and release of IFNγ expression measured after 18 h of incubation using ELISPOT (enzyme-linked immune absorbent spot). The values are represented as stimulation indexes (SIs) relative to the DMSO control. As positive control, the benchmark CEFT peptide pool (0.5 μM) was used, which induced significant CD4+ T cell activation in all donors tested. For statistical analysis, a modified-distribution free resampling (DFRlp) was utilized. DFRlp permutation resampling allows for a maximum false-positive rating of 5% (p ≤ 0.05) and a minimum of 1% (p ≤ 0.01).

FVIII Inhibitor/Neutralizing Antibody Assay
Plasma anti-FVIII IgGs were quantified by ELISA.57 FVIII inhibitor titer was determined by modified Bethesda assay.57 A FVIII monoclonal antibody (GMA-8021) with defined titers was used as standard. Rat antibodies against BDD-FVIII-X5 were generated by injecting an AAV8-FVIII-X5 vector into HA rats. Inhibitor-containing plasma was collected, hFVIII-specific antibodies were absorbed by an excess of biotinylated hFVIII, and the resulting biotin-FVIII-inhibitor complexes were removed by streptavidin agarose. Biotinylated BSA was used as non-binding control. Resulting plasma samples were used to assay for X5-specific FVIII inhibitors using BDD-FVIII-X5 as target.
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AUTHOR CONTRIBUTIONS
W.C., B.D., F.H., J.L., B.M.R., H.R., and W.X. designed research and analyzed data; W.C., B.D., J.A.F., A.R.M., Q.W., W.W., H.W., and S.A.R. performed experiments; W.C, C.J.B., J.A.F., H.R., and W.X. wrote the paper. D.L., C.J.B., B.K., M.d.I.R., F.S., and C.M. contributed key reagents and helpful comments and discussion.

DECLARATION OF INTERESTS
A patent application has been submitted by W.X., B.D., and W.C. for contributed key reagents and helpful comments and discussion.

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