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Identification of Key Coagulation Activity Determining Elements in Canine Factor VIII

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It is well known that canine factor VIII (cFVIII) has a higher specific activity than does human FVIII (hFVIII), and it has been previously demonstrated that cFVIII light chain is able to enhance hFVIII activity. The goal of this study was to first determine which amino acids in cFVIII light chain were responsible for enhancing hFVIII activity, and second to use these amino acids to develop a hFVIII variant with enhanced functional activity. We systemically screened segments of cFVIII light chain by testing an array of human-canine light chain hybrids and found that canine amino acids 1857–2147 were key to this enhancement. Each canine amino acid within this span was screened individually using a negative selection method, which led to the identification of 12 aa (JF12) in the FVIII light chain that could enhance activity. Substitution of the corresponding 12 aa into hFVIII (hFVIIIJF12BDD) elevated the specific activity profile *in vitro*. Furthermore, hFVIIIJF12BDD expressed an *in vivo*-displayed increased coagulation activity compared to wild-type, while maintaining normal secretion efficiency. In conclusion, we identified the amino acids in cFVIII that are the key determinants for higher specific activity and may be the basis for future development of therapeutic treatments for hemophilia A.

INTRODUCTION

Hemophilia A (HA) is an X-linked genetic disorder caused by pathogenic variants in the *F8* gene resulting in the production of either no factor FVIII (FVIII) protein or a nonfunctional or dysfunctional FVIII protein.[1–3] Currently, the standard treatment of HA relies on the prophylactic intravenous (i.v.) infusion of recombinant or plasma-derived FVIII protein.[4,5] While this replacement treatment corrects the abnormal bleeding phenotype, it is life-long and time-consuming[6] and is estimated to cost from $150,000 to $300,000 per patient per year in the United States.[7] Therefore, the development of a FVIII protein with increased activity would be valuable and could potentially enhance the quality of life for HA patients.

The concept that a more effective FVIII protein could be developed came from the observation that multiple FVIII orthologs have superior clotting profiles compared to human FVIII (hFVIII).[8,9] FVIII protein from pigs, dogs, mice, and monkeys has been tested and was revealed to function appropriately in the human clotting cascade and have the ability to bind human von Willebrand factor (vWF),[10] yet also display different biochemical profiles. For example, recombinant ovine FVIII with the B domain deleted has a greater specific activity, and longer half-life following activation, compared to its human counterpart.[10,11] Porcine FVIII has been demonstrated to secrete 10- to 100-fold more efficiently compared to hFVIII,[11,12] and recently a recombinant porcine FVIII was approved for the treatment of acquired HA.[13] Recombinant canine FVIII (cFVIIIBDD) has a higher specific activity compared to its human counterpart.[11,14,15] However, the direct use of these orthologs in normal patients, without inhibitors, is considered disadvantageous due to the possibility of an immune response. Since the etiology of inhibitor development is unclear,[16] changes to amino acid sequence and protein structure are avoided. Therefore, determining the amino acids responsible for the beneficial properties of these orthologs would be valuable, in terms of developing a modified hFVIII construct that has increased coagulation activity.

Previously, it was reported that cFVIIIBDD is 3- to 7-fold more active compared to B domain-deleted hFVIII (hFVIIIBDD).[11,14] The observed increase in specific activity was predominantly due to the canine light chain (cLC) sequence,[11] which was confirmed *in vitro*, and from dual delivery of AAV carrying FVIII human heavy chain (hHC) and cLC to HA mice *in vivo*.[11] In the current study, the amino acids in cFVIII LC were systemically evaluated for their ability to enhance hFVIII activity. To begin, human and canine hybrid constructs containing different spans of canine and human amino acid sequences were generated in order to ascertain the portion of cLC that is responsible for the observed increase in FVIII activity. Next, the canine amino acids within this region were individually analyzed for their ability to enhance hFVIII activity using a process of negative

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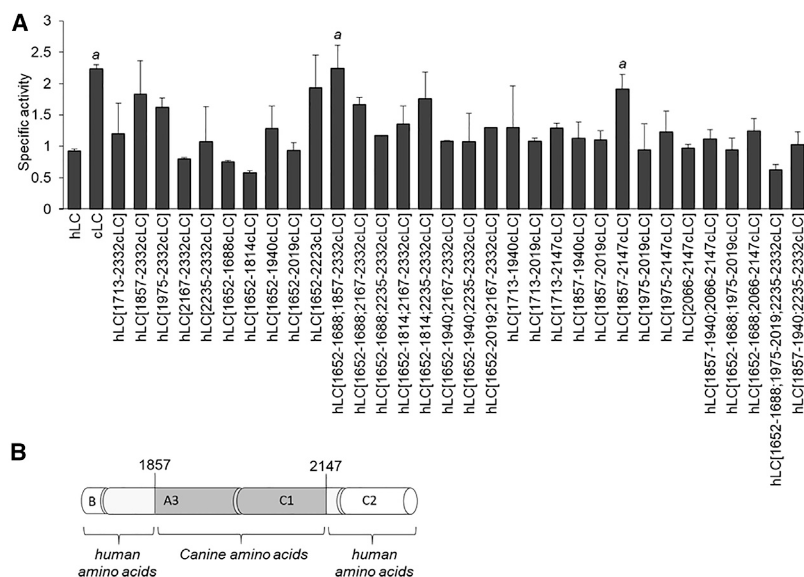

Figure 1. Function of hFVIII LC Hybrids *In Vitro*

HEK293 cells were transiently transfected with hHC and hLC/cLC hybrid plasmids at a ratio of 1:2 (LC/HC). Statistical analysis was performed using a two-tailed Student's t test. Differences were considered significant when $p < 0.05$. Error bars represent standard deviation. (A) Specific activity of each hybrid construct. Specific activity is determined by comparing coagulation activity to the amount of protein secreted (U/mL APTT/U/mL HC secreted). Constructs that were significantly different from hLC, but not significantly different from cLC, are labeled with the letter *a.* (B) Schematic diagram of construct hLC[1857–2147cLC].

selection. This led to the identification of a unique 12-aa sequence that was incorporated into hFVIII. The resulting variant, hFVIIIBDDJF12, exhibited enhanced FVIII specific activity *in vitro* and *in vivo*. Based on these results, hFVIIIBDDJF12 has potential for use in both protein replacement therapy and gene therapy.

RESULTS

Canine Amino Acids 1857–2047 Enhance FVIII Activity *In Vitro* and *In Vivo*

Preliminary testing *in vitro* confirmed that the cLC was able to increase hFVIII activity (Figure S1). Prompted by this observation, we set out to determine which amino acids in the cLC increased FVIII activity. This was accomplished using eight sets of primers designed based on areas of shared nucleotide sequence between hLC and cLC (Figure S2) to create 33 human/canine hybrid constructs (Table S1) that contained different portions of human and canine amino acid sequences. Constructs were expressed *in vitro* through transfection of HEK293 cells using a dual-chain delivery method,[17] tested for activity using a one-stage activated partial thromboplastin time (APTT) assay, and protein was measured using an ELISA detecting hHC (Figures S3A and S3B).

Based on these results, the specific activity was calculated by comparing construct activity (U/mL FVIII determined by APTT) to protein amount (ng of hHC/mL quantified by ELISA) (Figure 1). Only two constructs were identified that had activity similar to that of cLC, constructs hLC[1652–1688;1857–2332cLC] and hLC[1857–2147cLC]. Since both of these constructs contained canine amino acid sequences from amino acids 1857–2147, this region of canine sequence was considered positively correlated with enhanced activity, and construct hLC[1857–2147cLC] was selected for further studies (Figure 1B).

Next, the activity of hLC[1857–2147cLC] was tested *in vivo* through hydrodynamic injection of HA mice with plasmid DNA coding for hHC and either hLC, cLC, or hLC[1857–2147cLC]. Results found that mice injected with hHC and either cLC or hLC[1857–2147cLC] had significantly higher coagulation activity compared to mice injected with hHC and hLC, as determined by a one-stage APTT assay, yet there was no significant difference in protein amount (Figures 2A and 2B). The calculated specific activity was significantly higher for mice injected with hHC and either cLC or hLC[1857–2147cLC] compared to mice injected with hHC and hLC (Figure 2C). There was no significant difference between cLC and hLC[1857–2147cLC] for any of the analyses performed.

Identification of Key Amino Acids in hLC[1857–2147cLC] through Negative Selection

Alignment of amino acid sequences for hLC[1857–2147cLC] and hLC revealed a 26-aa difference (Figure S4). It was hypothesized that not all of these amino acids were necessary to enhance FVIII activity. Based on this supposition, each canine amino acid in construct hLC[1857–2147cLC] was evaluated individually. This was accomplished using a process of negative selection, in which mutant constructs were designed to carry the hLC[1857–2147cLC] sequence with one amino acid switched back to the original human amino acid (Table S3). If the amino acid contributed significantly to the increase in FVIII activity, removing it should result in a decrease in activity. Each mutant was tested *in vitro* through co-transfection of HEK293 cells with hHC, and activity was determined using a one-stage APTT assay and calculated as percent hLC[1857–2147cLC] activity (Figure 3). In this experiment, protein amount was not measured because previous testing demonstrated that hLC[1857–2147cLC] did not alter protein levels (Figure S3). The majority of mutants tested (n = 13) fell within the range of 70%–100% hLC[18547–2147cLC] activity. However, 10 mutants demonstrated less than 70% activity, which indicated that canine amino acid was essential for increasing FVIII activity. Based on these results, the hLC-JF10 construct was designed as a hLC that contained these 10 canine aa (Figure S5).

Development of a hFVIII Variant with Enhanced FVIII Activity

During the cloning process for hLC-JF10, potential constructs were screened for activity using a one-stage APTT assay. One clone was

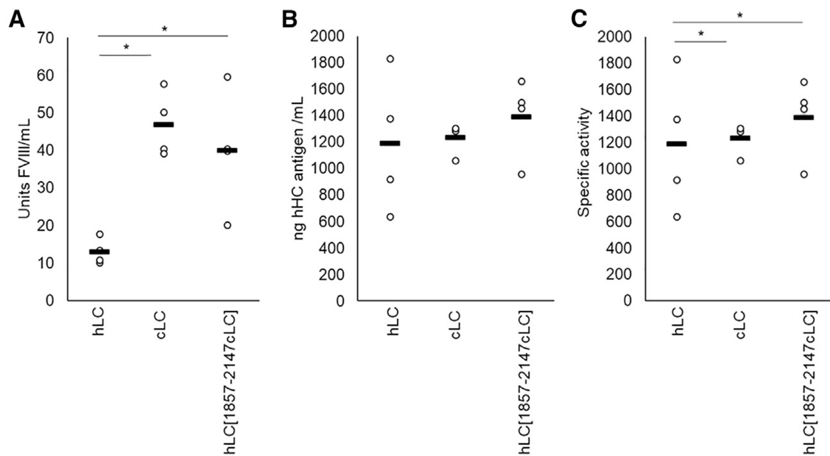

Figure 2. Canine Amino Acids 1847–2147 Enhance FVIII Activity *In Vivo*

Three groups of HA mice were hydrodynamically injected with 150 μg of plasmid DNA at a 1:1 ratio (HC/LC). Blood was harvested 48 h post-injection from the hepatic artery. Statistical analysis was performed using a two-tailed Student's t test. *p < 0.05. (A) U/mL FVIII produced from each group determined by an APTT assay. (B) ng of HC antigen/mL detected by an ELISA for hHC antigen. (C) Specific activity of FVIII produced. Specific activity is determined by comparing coagulation activity to the amount of protein secreted (U/mL APTT/U/mL HC secreted).

found to have activity superior to that of cLC and hLC[1857–2147cLC] (Figure S6). Sequencing of this clone revealed two exogenous mutations in the C1 domain, S2157N and R2159H, that were neither human nor canine amino acids (Figure S7). These were unexpected and random mutations, which produced intriguing results. This hLC construct that had 10 canine aa and 2 exogenous aa was named hLC-JF12. In order to determine whether the 2 exogenous aa had an impact on activity, hLC-X2 was engineered as a hLC construct that carried only the 2 exogenous aa mutations (Table 1; Table S5).

Testing *in vitro* expectedly revealed that cLC, hLC[1857–2147cLC], and hLC-JF10 had significantly higher coagulation activity compared to hLC, but none of these could achieve the same level of activity as hLC-JF12 (Figure 4A). Interestingly, hLC-JF12 also resulted in a significant increase in protein amount, where other constructs did not significantly affect protein levels compared to hLC (Figure 4B). The specific activity was calculated for each construct, which demonstrated that cLC, hLC[1857–2147cLC], hLC-JF10, and hLC-JF12 all had a significantly higher specific activity compared to hLC; however, hLC-JF12 had the highest activity of all of these variants (Figure 4C). Construct hLC-X2 was not significantly different from hLC in any of the assays. Based on these results it was evident that without the X2 mutations, hLC-JF10 was unable to achieve activity to the same level as hLC-JF12; however, the X2 mutations alone did not confer any changes to activity. Taken together, it was concluded that all 12 aa changes were required for the superior activity.

Next, the hLC-JF12 construct was tested *in vivo* using an AAV vector for delivery. The hHC, hLC, cLC, and hLC-JF12 sequences were packaged into a single-stranded AAV8 vector and delivered along with an AAV8 vector containing hHC via tail vein injection to 6-week-old male CD4KO/HA mice, and blood was harvested via retro-orbital eye bleeding for 12 weeks. The results of a one-stage APTT assay demonstrated that both the AAV8-cLC and AAV8-JF12 constructs were able to significantly increase coagulation activity at 2, 4, and 6 weeks post-injection (Figure 5A). At 8 and 12 weeks post-injection, the AAV8-cLC and AAV8-JF12 groups had higher coagulation activity, but this did not reach significance due to variation. An ELISA detecting hHC antigen demonstrated that there was no significant difference in the amount of protein present in any group (Figure 5B). An ELISA detecting hLC confirmed that there was no significant difference in protein amounts between the hLC and the hLC-JF12 groups (Figure 5C).

Single-Stranded FVIII Carrying the JF12 Modifications Enhances Coagulation Activity *In Vitro* and *In Vivo*

Single-stranded hFVIIIBDD constructs were engineered to contain either the JF12 mutations (hFVIIIJF12BDD) or the canine amino acids 1857–2147 (hFVIIIhLC[1857–2147cLC]BDD). *In vitro* testing revealed that the hFVIIIhLC[1857–2147cLC]BDD construct increased coagulation activity by ~2.8-fold, while the JF12 modification resulted in an ~8.8-fold increase in coagulation activity (Figure 6A). For single-chain delivery, both hFVIII[1857–2147cLC] and hFVIIIJF12BDD had a significant increase in protein amount as measured by ELISA, although hFVIIIJF12BDD was significantly higher than both hFVIIIBDD and hFVIII[1857–2147cLC]BDD (Figure 6B). Both hFVIII[1857–2147cLC]BDD and hFVIIIJF12BDD had significantly higher specific activity compared to hFVIIIBDD, but they were not significantly different from each other (Figure 6C). Next, hFVIIIJF12BDD and the hFVIIIBDD constructs were packaged into single-stranded AAV8 vectors, resulting in AAV8-hFVIIIJF12BDD and AAV8-hFVIIIBDD. These vectors were delivered to CD4KO/HA mice via tail vein injection, and blood was harvested through retro-orbital eye bleeding during the course of 12 weeks. The results of a one-stage APTT test demonstrated that the AAV8-hFVIIIJF12BDD construct produced a significantly higher amount of coagulation activity compared to AAV8-hFVIIIBDD for every time point tested (Figure 6D). An ELISA detecting hHC demonstrated that, except for week 2 after injection, there was no significant increase in protein amount for AAV8-hFVIIIJF12BDD compared to AAV8-hFVIIIBDD (Figure 6E). This indicates that the JF12 modifications produce a FVIII protein with higher coagulation activity and have no effect on protein secretion.

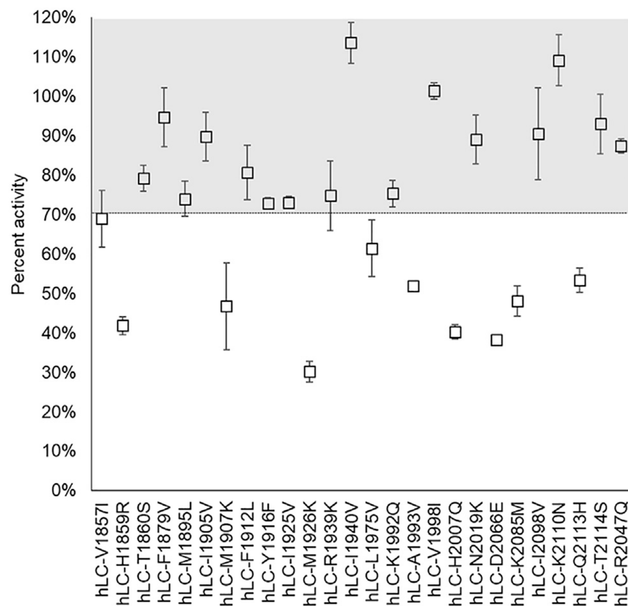

Figure 3. Identification of Canine Amino Acids that Enhance hLC[1857–2147cLC] Activity

A total of 26 hLC[1857–2147cLC] mutants were engineered to contain the hLC [1857–2147cLC] sequence with one of the canine amino acids reverted back to its human counterpart. Each mutant was tested by transfection of HEK293 cells with hHC at a ratio of 1:2 (LC/HC). An APTT assay was used to determine activity in U of FVIII/mL. The activity for each mutant was divided by the activity of hLC[1857–2147cLC] to calculate percentage activity. Mutants that demonstrated less than 70% hLC[1857–2147cLC] activity were identified. Statistical analysis was performed using a two-tailed Student's t test. $^{*}p < 0.05$. Error bars represent standard deviation.

Table 1. Amino Acid Changes in LC Constructs JF10, JF12, and X2

Amino Acid	Name	U of FVIII/mL	Percent Activity	ng of hHC Antigen	Specific Activity
1857–2147	hLC[1857–2147kLC]	0.53	100.0	33.88	1.56
1857	hLC-V1857I	0.39	73.2	30.65	1.26
1859	hLC-H1859R	0.21	39.9	20.84	1.00
1860	hLC-T1860S	0.37	71.0	22.90	1.66
1879	hLC-F1879V	0.50	95.2	24.37	2.05
1895	hLC-M1895L	0.37	70.5	26.95	1.41
1905	hLC-I1905V	0.47	90.2	35.93	1.45
1907	hLC-M1907K	0.23	43.8	24.05	0.96
1912	hLC-F1912L	0.43	81.2	28.50	1.50
1916	hLC-Y1916F	0.43	81.7	35.25	1.19
1925	hLC-I1925V	0.39	73.4	32.51	1.23
1926	hLC-M1926K	0.17	31.9	16.16	1.04
1939	hLC-R1939K	0.42	79.6	34.71	1.29
1940	hLC-I1940V	0.59	113.0	32.90	1.80
1975	hLC-L1975V	0.37	69.7	27.65	1.38
1992	hLC-K1992Q	0.40	75.6	28.00	1.43
*1993	hLC-A1993V	0.36	69.3	31.41	1.22
1998	hLC-V1998I	0.58	110.2	41.96	1.43
2007	hLC-H2007Q	0.29	56.0	24.46	1.18
2019	hLC-N2019K	0.41	77.3	37.80	1.13
2085	hLC-K2085M	0.26	49.9	26.27	1.00
2098	hLC-I2098V	0.40	76.0	31.16	1.30
2110	hLC-K2110N	0.58	109.5	38.36	1.53
2113	hLC-Q2113H	0.30	56.7	20.86	1.43
2114	hLC-T2114S	0.51	96.6	32.30	1.57
2147	hLC-R2047Q	0.43	80.9	26.33	1.64

The FVIII hLC was designed to contain 10 canine aa (hLC-JF10), 10 canine aa plus 2 exogenous aa changes (hLC-JF12), or 2 exogenous aa changes (hLC-X2).

The hFVIIIJF12BDD Protein Had Increased Specific Activity and Generated FXa More Robustly Compared to hFVIIIBDD

In order to gain insight into how the JF12 modifications increased FVIII specific activity, protein was partially purified using a SP Sepharose high-performance strong cation exchange column. The results of a western blot indicated that there were no differences in the banding patterns between hFVIIIBDD and hFVIIIJF12BDD, which demonstrated that the JF12 modifications do not affect chain size or heterodimer formation (Figure S8A). Next, the specific activity of hFVIIIJF12BDD was determined by comparing the amount of protein, quantified by ELISA, to the activity produced by a one-stage APTT assay. The results showed that the hFVIIIJF12BDD protein produced a specific activity of 39,153.69 U/mg compared to hFVIIIBDD, which only had a specific activity of 6,247.92 U/mg (Figure S8B). These results verified the *in vivo* observation that the JF12 modifications increase protein specific activity.

Finally, a FXa generation test demonstrated that the hFVIIIJF12BDD protein was able to convert FX to FXa more efficiently compared to hFVIIIBDD (Figure S8C). Using a Michaelis-Menten kinetics analysis, the K_m (substrate concentration at one-half the maximum velocity) and V_{max} (maximum velocity) for hFVIIIJF12BDD and hFVIIIBDD were determined. The K_m for hFVIIIJF12BDD was calculated at 24.29, compared to 29.14 for hFVIIIBDD, showing that the tenase complex formed from hFVIIIJF12BDD binds to FX with a higher affinity (Figure S8D). The V_{max} for hFVIIIJF12BDD was 11.93, which is lower compared to hFVIIIBDD, with a V_{max} of 15.33 (Figure S8D). This indicated that the hFVIIIJF12BDD construct converts FX to FXa at a quicker rate.

DISCUSSION

It is highly desirable to improve the hFVIII coagulation profile through bioengineering, and a number of FVIII variants with altered properties have been previously described.[18–22] Herein, we present the first study to systemically identify key amino acids that may improve hFVIII specific activity utilizing cFVIII as the starting template, which has been documented for its higher specific activity. This strategy avoids the use of random mutations that may disrupt the structure entirely and focuses on the differences that may confer the better properties

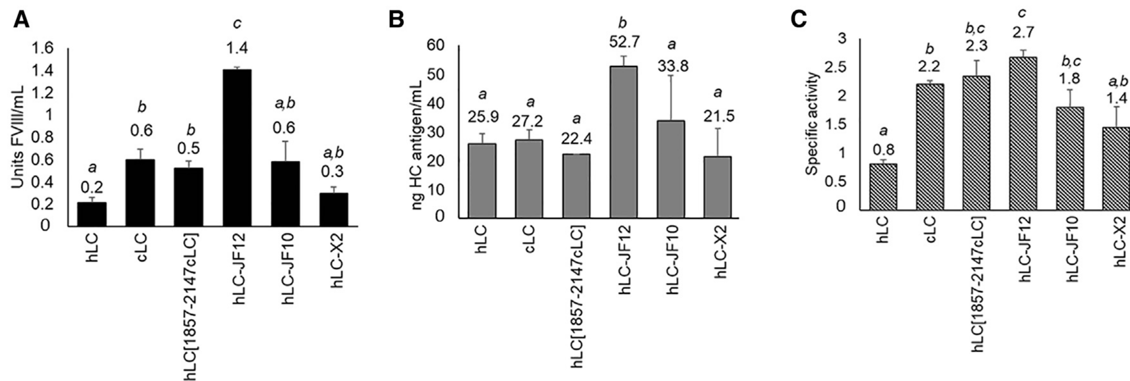

Figure 4. Functional Properties of FVIII Human Hybrid Constructs Tested *In Vitro*

Constructs hLC-JF12, hLC-JF10, and hLC-X2 were tested and compared to hLC, cLC, and hLC[1857–2147cLC] through transfection of HEK293 cells at a plasmid ratio of 1:2 (LC/HC). Statistical analysis was performed using a two-tailed Student's t test. Differences were considered significant when $p < 0.05$. Groups labeled with similar letters are not significantly different from each other. Error bars represent standard deviation. (A) U of FVIII/mL produced by each hybrid, determined via an APTT assay. (B) ng of hHC antigen/mL detected by ELISA for each hybrid construct. (C) Specific activity of each hybrid. Specific activity is determined by comparing coagulation activity to amount of protein secreted (U/mL APTT/ng of HC antigen secreted).

observed experimentally. The methods can be expanded to include other FVIII orthologs, selecting for different functional properties.

In the present work, we found that canine amino acids within the 1857–2147 coding region were able to elicit an increase in FVIII specific activity. Based on this information, a 12-aa modification was developed (JF12) that, when integrated into hFVIII, produced superior clotting activity *in vitro* and through AAV gene delivery *in vivo* when tested using the dual-chain delivery method and as a single-chain vector. Importantly, data from AAV injections clearly proved that the JF12 modifications increased coagulation activity and not protein amount, and, by doing so, they increased the therapeutic value of the protein itself. This is most clear with the single-stranded vector delivery (Figures 6D and 6E), where the amount of protein present is quite low for mice injected with either AAV8-hFVIIIBDD or AAV8-hFVIIIJF12BDD due to the intentional use of a low vector titer. Using a lower amount of vector allowed us to observe how the JF12 modifications would function when only a small amount of protein is present. In this experiment, peak expression for mice injected with AAV8-hFVIIIBDD averaged 6.1% FVIII activity at week 6, whereas peak expression for mice injected with AAV8-hFVIIIJF12BDD averaged 21.3% at week 8. There was no statistical difference between the amounts of protein present between AAV8-hFVIIIBDD and AAV8-hFVIIIJF12BDD at either of these time points. These results illustrated that the JF12 modifications are able to increase the level of activity, and even when the amount of protein is low, activity remains above therapeutic value.

In order to determine how these 12 aa functioned to enhance activity, protein was partially purified and analyzed. The results of testing using this protein found that the JF12 modifications do not affect FVIII structure. Functional testing found that the hFVIIIJF12BDD protein has a higher specific activity compared to hFVIIIBDD, validating the hypothesis that the JF12 modifications increase FVIII specific activity. Basic functional testing was performed in order to try and explain the observed phenomenon and found that the hFVIIIJF12BDD protein was able to convert FX to FXa more efficiently compared to hFVIIIBDD by binding to FX with a higher affinity and converting FX to FXa at a quicker rate. However, the recorded increases were not enough to fully explain the enhancing effects of the JF12 modifications. Previously it was reported that changes to the FVIII C1 spike (amino acids 2158–2159) may also alter protein interactions and cofactor activity.[23] In this case it could be argued that JF12 was beneficial in terms of cofactor activity, due to the increase in FXa generation. Conversely, changes to amino acids 2157 and 2159 may affect vWF binding.[24] These analyses, and further functional testing, are required to detail the pharmacokinetics of the hFVIIIJF12BDD protein and to understand the mechanism behind the enhanced activity to a fuller extent.

In this study, we identified that a total of 12 aa changes are necessary for enhanced specific activity. The results suggested that coordinated action of these amino acids is necessary for the improvement. While this variant provides new insight into the structure and function of factor VIII, the potential of this variant for therapeutic use may be explored under special situations. Note that whether these modifications may have an effect on inhibitor formation has not been tested. Inhibitors are primarily immunoglobulin G that can develop in up to 30% of patients receiving treatment and are considered to form predominantly against epitopes in the A2 and C2 domains.[25,26] The fact that the 12 aa changes described herein fall within the A3 (1690–2019) to C1 (2020–2172) domain[27] suggests that it may not affect antibody formation. However, recent data have suggested that residues within the C1 and C2 domain are associated with the uptake of FVIII by dendritic cells, which can elicit an immune response.[28] In addition, amino acid 2085 is located on a peptide that is frequently associated with histocompatibility leukocyte antigen (HLA) molecules,[29] and the two *de novo* mutations 2157 and 2159 are located in a T cell epitope.[30] Therefore, whether the JF12 modifications affect

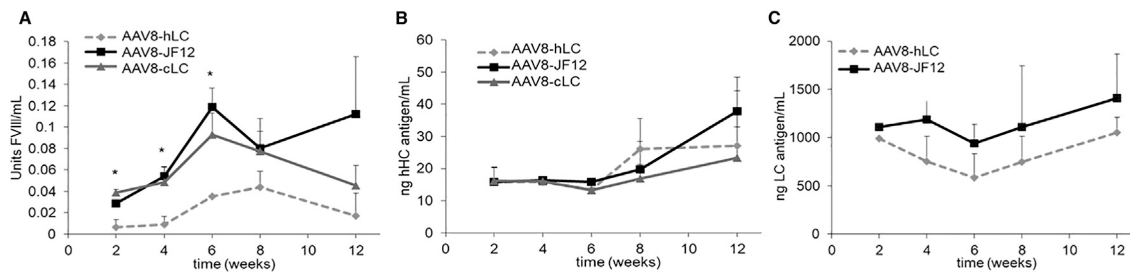

Figure 5. Construct JF12 Has Superior Coagulation Activity *In Vivo* When Delivered Using a Dual-Chain AAV Vector Strategy

AAV8 vector was packaged with hHC and hLC, cLC, or hLC-JF12 and delivered to CD4KO/HA mice using a dual-chain delivery strategy. Blood was harvested via retro-orbital bleeding during the course of 3 months. Statistical analysis was performed using a two-tailed Student's t test, and differences were considered significant when $p < 0.05$. *$p < 0.05$, for differences between AAV8-cLC or AAV8-JF12 and AAV8-hLC. Error bars represent standard deviation. (A) U of FVIII/mL produced by each hybrid, determined via an APTT assay. (B) ng of hHC antigen/mL detected by ELISA for each hybrid construct. (C) ng of LC antigen/mL detected by ELISA for hLC- and hLC-JF12-injected groups.

inhibitor formation or any other immunological properties will require further evaluation.

MATERIALS AND METHODS

Engineering Human/Canine Hybrids and Mutant Constructs

All human/canine hybrid constructs and single-chain constructs were engineered using primers (Integrated DNA Technologies) that annealed to areas of a shared nucleotide sequence. The list of hybrid constructs can be found in Table S1, and primers used are found in Tables S2 and S3. The hLC[1857–2147cLC] mutants and hLC-J10 and hLC-X2 were engineered using primers containing the nucleotide sequences of interest; primer sequences can be found in Tables S4 and S5. Using the specified primers, constructs were cloned according to the following protocol: PCR amplification (Biometra thermocycler) was run for 35 cycles using either Vent polymerase (New England Biolabs) or Taq polymerase (Sigma-Aldrich). Fragments were mixed together at a molar ratio of 1:1, heated to 95°C (denaturation), and cooled to 55°C (annealing). The single-stranded regions were filled in using Vent polymerase and 10 mM deoxyribonucleotide triphosphate (DNTP) mix (Sigma-Aldrich). These sequences were used as a template for PCR amplification using Vent polymerase. After amplification, the product was digested with Not1 and BstB1, purified on a 0.8% agarose gel (Thermo Fisher Scientific gel extraction kit), and inserted into an adeno-associated virus (AAV) plasmid backbone using a Ligation kit (New England Biolabs).

For the initial screening process, hybrids were engineered as LC constructs that were tested in conjugation with the hHC using the dual-chain delivery method. In this way, each hybrid LC was tested using the same hHC construct. After screening was complete and the construct of interest identified, a single-stranded FVIII hybrid was created (hFVIIIJF12BDD). For engineering the full-length FVIII construct, the B domain-deleted version of FVIII was utilized due to its smaller size and activity profile.

Cellular Transfection Method

HEK293 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen), supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin antibiotics (Invitrogen), and maintained at 37°C, supplemented with 5% CO_2. To test constructs, cells at 80%–100% confluency were transiently transfected using PolyJet (SignaGen Laboratories). For dual-chain transfection, LC and HC plasmids were transfected using a 1:2 ratio. Transfection was maintained for 16 h, after which cells were washed with Ham's FXIII media (Gibco), followed by the addition of a total of 400 μL of Ham's FXII media, supplemented with 2% heat-inactivated FBS.

AAV Vector Preparation

AAV vectors were serotype 8 and produced following the well-described triple-plasmid transfection method.[31,32] After purification by cesium chloride gradient ultracentrifugation, they were buffer exchanged against PBS with 5% D-sorbitol. Vector purity and genome titer were determined by silver staining (Pierce silver stain kit) and qPCR using primers targeting the FVIII HC.

Partial Protein Purification

Protein was produced by transfection of HEK293 cells using PolyJet reagent. After transfection, media were collected and filtered using a 0.2-μm Whatman filter, 2-(*N*-morpholino)ethanesulfonic acid (MES) was added to a final concentration of 20 mM, and pH was adjusted to 6.0. The protein was partially purified using a SP Sepharose high-performance strong cation exchange column (HiTrap SP HP; GE Healthcare) pre-equilibrated with equilibration/washing buffer (10 mM MES, 20 mM $CaCl_2$, 200 mM NaCl, 0.01% Tween 80, pH 6.0). The column was washed with a 5-column vol of washing buffer, and protein was eluted using a linear increase of sodium concentration up to 1 M. The elution was monitored using a ÄKTAFPLC system (GE Healthcare), and fractions with the highest clotting activity, determined by a one-stage APTT clotting assay, were pooled together and concentrated by an Amicon concentrator (molecular weight cut-off of 50,000 Da) (Millipore). The purified protein was visualized using SDS-PAGE electrophoresis.

In Vivo Testing of Constructs

C57BL6/129sv/HA and CD4KO/HA mice were bred and maintained in a pathogen-free environment supplied with a normal

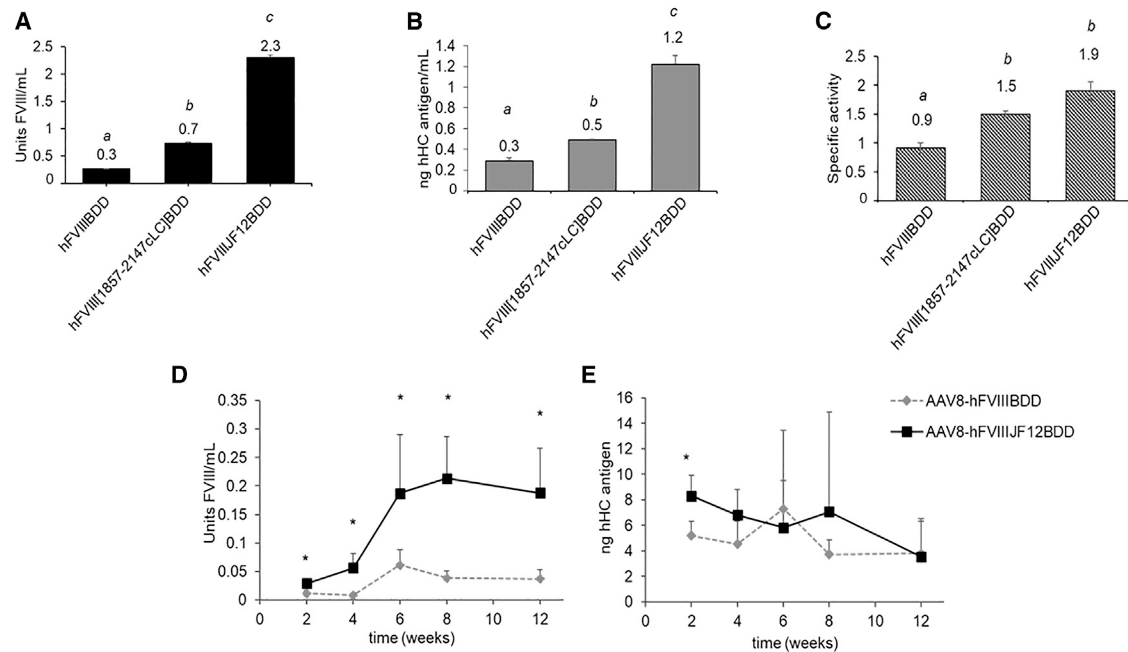

Figure 6. Canine Amino Acids from 1857–2147 and the JF12 Modifications When Delivered as a Single-Chain FVIII Molecule

(A–C) Single-chain hFVIIIBDD constructs were designed to contain either canine amino acids 1857–2147 (hFVIII[1857–2147cLC]BDD) or the JF12 modifications (hFVIIIJF12BDD) and tested *in vitro* through transfection of HEK293 cells. Statistical analysis was performed using a two-tailed Student's t test, and differences were considered significant when $p < 0.05$. Groups labeled with similar letters are not significantly different from each other. Error bars represent standard deviation. (A) U of FVIII/mL produced by each hybrid, determined via an APTT assay. (B) ng of hHC antigen/mL detected by ELISA for each hybrid construct. (C) Specific activity of each hybrid. Specific activity is determined by comparing coagulation activity to amount of protein secreted (U/mL APTT/ng of HC antigen secreted). (D and E) The hFVIIIJF12BDD sequence was packaged into an AAV8 vector (AAV8-hFVIIIJF12BDD) and delivered to CD4KO/HA mice. Mice injected with AV8-hFVIIIBDD served as the control. Blood was harvested via retro-orbital bleeding during the course of 3 months. Statistical analysis was performed using a two-tailed Student's t test, and differences were considered significant when $p < 0.05$. *$p < 0.05$, for differences between AAV8-cLC or AAV8-JF12 and AAV8-hLC. (D) U of FVIII/mL produced by each hybrid, determined via an APTT assay. (E) ng of hHC antigen/mL detected by ELISA for each hybrid construct.

diet. All procedures were carried out in accordance with institutional guidelines under approved protocols at Temple University Medical School. Hydrodynamic injections were performed on 12-week-old C57BL6/129sv/HA mice by delivering 150 μg of plasmid DNA in a total volume of 2 mL of 0.9% NaCl solution via the tail vein within 10 s. After 48 h, mice were sacrificed following standard euthanasia procedures, and blood was harvested via the hepatic artery and collected into 0.4% sodium citrate solution. AAV vector was diluted in 0.9% NaCl solution to a total volume of 200 μL (1–4 $\times$ 10^{11} vector particles [vp]/mouse) and injected via the tail vein of 6- to 8-week-old CD4KO/HA mice. Following injections, blood was harvested every 2–4 weeks via retro-orbital eye bleeding and collected in 0.4% sodium citrate.

FVIII Clotting Activity and Quantification of FVIII Protein by ELISA

FVIII clotting activity was determined for each hybrid tested using a one-stage APTT assay. In short, 50 μL of sample, 50 μL of FVIII-deficient plasma (Haematologic Technologies), and 50 μL of APTT reagent (PTT Automate 5; Diagnostica Stago) were mixed in a standard APTT cuvette (Diagnostica Stago) and incubated for 170 s at 37°C. Afterward, 50 μL of 25 mM $CaCl_2$ was abruptly added, and the time until clot formation was measured. FVIII protein Kogenate (Bayer Healthcare) was used as the standard. An ELISA was used to quantify FVIII antigen. Briefly, plates were coated with hHC antigen GMA-8016 or human LC antigen GMA-8018 (Green Mountain Antibodies), stored overnight at 4°C, and then replaced with 3% BSA in phosphate-buffered saline with Tween 20 (PBST) and incubated. Then, wells were washed with PBST, and secondary antibody was added; HC was GMA-8015 biotinylated and LC was GMA-8022 biotinylated. After the secondary antibodies were removed, wells were washed with PBST, and horseradish peroxidase was added. Then, wells were washed and developed using a KPL tetramethylbenzidine (TMB) microwell peroxidase substrate system. The plate was read at 450 nm using a Molecular Devices Thermomax microplate reader. Kogenate FS (Bayer Healthcare) was used as the standard.

Western Blot Analysis

Protein samples were combined with NuPage dye containing 50 mM DTT, loaded onto an 8% SDS-PAGE gel, and run for 1 h at 180 V. Then, the protein was transferred to a nitrocellulose membrane. After transfer, the membrane was washed in PBST, and 5% non-fat milk in PBST was added. After 1 h, the membrane was washed and incubated with a polyclonal sheep anti-hFVIII antibody (Haematologic

Technologies). Next, the membrane was washed and incubated with a IRDye 800CW-conjugated donkey anti-sheep IgG (immunoglobulin G) (H+L) (Rockland). An Odyssey infrared imaging system (LI-COR Biosciences) was used to scan the membrane.

Protein Activity

The specific activity was determined using the following steps: the hFVIIIBDD and hFVIIIJF12BDD proteins were purified in the manner described above. An ELISA was performed to quantify final FVIII protein using antibodies for the hHC antigen. After quantification, protein was diluted to a concentration of 100 ng/mL in HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer supplemented with 1% BSA. A one-stage APTT assay was used to determine coagulation activity. The specific activity was calculated as U of APTT activity per mg of protein (U/mg). A FXa generation assay was used to further analyze protein activity. To begin, the phosphatidylcholine (PC)/phosphatidylserine (PS) (7:3, w/w) phospholipid vesicle was prepared and 1 nM hFVIIIBDD or hFVIIIJF12BDD was activated by 10 nM thrombin at 37°C for 2 min in the presence of 5 mM $CaCl_2$. The reaction was stopped by adding 5 U of hirudine. 40 nM FIXa was included in reaction mixture for 1 min to allow for the tenase complex to form. The FXa generation reaction was started by adding different concentrations of FX at 37°C, and the reaction was terminated 2 min later by 10 mM EDTA. The FXa generated was measured by its hydrolysis of chromogenic substrate S2765 (350 μM). The rate of absorbance change was calculated with a standard curve generated with serially diluted FXa. The rate of FXa generation was plotted against FX concentration and curve was fitted with GraphPad software using the Michaelis-Menten equation to obtain K_m and V_{max}.

Statistical Analysis

Statistical analysis was performed using a two-tailed Student t test, based on the comparison of the same variable between two groups in an experiment. The difference between groups was considered significant when the p value was less 0.05 and not significant when the p value was more than 0.05. Error bars for each graph were derived using standard deviation from the average of at least three points.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2019.12.019.

AUTHOR CONTRIBUTIONS

J.F., Q.W., W.W., B.D., W.C., A.R.M., and S.R. performed experiments; B.A.K., C.M., D.L., L.L., and W.X. analyzed results. J.F., Q.W., W.W., B.D., and W.X. designed research. J.F. and W.X. wrote the paper.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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REFERENCES

1. Bowen, D.J. (2002). Haemophilia A and haemophilia B: molecular insights. MP, Mol. Pathol. *55*, 127–144.
2. Castaldo, G., D'Argenio, V., Nardiello, P., Zarrilli, F., Sanna, V., Rocino, A., Coppola, A., Di Minno, G., and Salvatore, F. (2007). Haemophilia A: molecular insights. Clin. Chem. Lab. Med. *45*, 450–461.
3. Doering, C.B., Healey, J.F., Parker, E.T., Barrow, R.T., and Lollar, P. (2002). High level expression of recombinant porcine coagulation factor VIII. J. Biol. Chem. *277*, 38345–38349.
4. Cafuir, L.A., and Kempton, C.L. (2017). Current and emerging factor VIII replacement products for hemophilia A. Ther. Adv. Hematol. *8*, 303–313.
5. Delignat, S., Peyron, I., El Ghazaly, M., V Kaveri, S., Rohde, J., Mueller, F., and Lacroix-Desmazes, S. (2018). Biochemical characterization and immunogenicity of Neureight, a recombinant full-length factor VIII produced by fed-batch process in disposable bioreactors. Cell. Immunol. *331*, 22–29.
6. Oldenburg, J. (2015). Optimal treatment strategies for hemophilia: achievements and limitations of current prophylactic regimens. Blood *125*, 2038–2044.
7. Armstrong, E.P., Malone, D.C., Krishnan, S., and Wessler, M.J. (2014). Costs and utilization of hemophilia A and B patients with and without inhibitors. J. Med. Econ. *17*, 798–802.
8. Zakas, P.M., Gangadharan, B., Almeida-Porada, G., Porada, C.D., Spencer, H.T., and Doering, C.B. (2012). Development and characterization of recombinant ovine coagulation factor VIII. PLoS ONE *7*, e49481.
9. Miao, H.Z., Sirachainan, N., Palmer, L., Kucab, P., Cunningham, M.A., Kaufman, R.J., and Pipe, S.W. (2004). Bioengineering of coagulation factor VIII for improved secretion. Blood *103*, 3412–3419.
10. Zakas, P.M., Vanijcharoenkarn, K., Markovitz, R.C., Meeks, S.L., and Doering, C.B. (2015). Expanding the ortholog approach for hemophilia treatment complicated by factor VIII inhibitors. J. Thromb. Haemost. *13*, 72–81.
11. Wang, Q., Dong, B., Firrman, J., Wu, W., Roberts, S., Moore, A.R., Liu, L.S., Chin, M.P., Diao, Y., Kost, J., and Xiao, W. (2016). Evaluation of the biological differences of canine and human factor VIII in gene delivery: implications in human hemophilia treatment. Gene Ther. *23*, 597–605.
12. Roberts, S.A., Dong, B., Firrman, J.A., Moore, A.R., Sang, N., and Xiao, W. (2011). Engineering factor Viii for hemophilia gene therapy. J. Genet. Syndr. Gene Ther. *1*, S1–S006.
13. Tarantino, M.D., Cuker, A., Hardesty, B., Roberts, J.C., and Sholzberg, M. (2017). Recombinant porcine sequence factor VIII (rpFVIII) for acquired haemophilia A: practical clinical experience of its use in seven patients. Haemophilia *23*, 25–32.
14. Sabatino, D.E., Freguia, C.F., Toso, R., Santos, A., Merricks, E.P., Kazazian, H.H., Jr., Nichols, T.C., Camire, R.M., and Arruda, V.R. (2009). Recombinant canine B-domain-deleted FVIII exhibits high specific activity and is safe in the canine hemophilia A model. Blood *114*, 4562–4565.
15. Staber, J.M., Pollpeter, M.J., Anderson, C.G., Burrascano, M., Cooney, A.L., Sinn, P.L., Rutkowski, D.T., Raschke, W.C., and McCray, P.B. (2017). Long-term correction of hemophilia A mice following lentiviral mediated delivery of an optimized canine factor VIII gene. Gene Ther. *24*, 742–748.
16. Lai, J.D., and Lillicrap, D. (2017). Factor VIII inhibitors: advances in basic and translational science. Int. J. Lab. Hematol. *39* (*Suppl 1*), 6–13.
17. Burton, M., Nakai, H., Colosi, P., Cunningham, J., Mitchell, R., and Couto, L. (1999). Coexpression of factor VIII heavy and light chain adeno-associated viral vectors produces biologically active protein. Proc. Natl. Acad. Sci. USA *96*, 12725–12730.
18. Mancuso, M.E., and Santagostino, E. (2017). Outcome of clinical trials with new extended half-life FVIII/IX concentrates. J. Clin. Med. *6*, E39.
19. Meunier, S., Alamelu, J., Ehrenforth, S., Hanabusa, H., Abdul Karim, F., Kavakli, K., Khodaie, M., Staber, J., Stasyshyn, O., Yee, D.L., and Rageliene, L. (2017). Safety and efficacy of a glycoPEGylated rFVIII (turoctocog alpha pegol, N8-GP) in paediatric patients with severe haemophilia A. Thromb. Haemost. *117*, 1705–1713.
20. Jiménez-Yuste, V., Lejniece, S., Klamroth, R., Suzuki, T., Santagostino, E., Karim, F.A., Saugstrup, T., and Møss, J. (2015). The pharmacokinetics of a B-domain truncated recombinant factor VIII, turoctocog alfa (NovoEight®), in patients with hemophilia A. J. Thromb. Haemost. *13*, 370–379.

21. Shestopal, S.A., Hao, J.J., Karnaukhova, E., Liang, Y., Ovanesov, M.V., Lin, M., Kurasawa, J.H., Lee, T.K., Mcvey, J.H., and Sarafanov, A.G. (2017). Expression and characterization of a codon-optimized blood coagulation factor VIII. J. Thromb. Haemost. *15*, 709–720.

22. Nguyen, G.N., George, L.A., Siner, J.I., Davidson, R.J., Zander, C.B., Zheng, X.L., Arruda, V.R., Camire, R.M., and Sabatino, D.E. (2017). Novel factor VIII variants with a modified furin cleavage site improve the efficacy of gene therapy for hemophilia A. J. Thromb. Haemost. *15*, 110–121.

23. Bloem, E., van den Biggelaar, M., Wroblewska, A., Voorberg, J., Faber, J.H., Kjalke, M., Stennicke, H.R., Mertens, K., and Meijer, A.B. (2013). Factor VIII C1 domain spikes 2092–2093 and 2158–2159 comprise regions that modulate cofactor function and cellular uptake. J. Biol. Chem. *288*, 29670–29679.

24. Chiu, P.L., Bou-Assaf, G.M., Chhabra, E.S., Chambers, M.G., Peters, R.T., Kulman, J.D., and Walz, T. (2015). Mapping the interaction between factor VIII and von Willebrand factor by electron microscopy and mass spectrometry. Blood *126*, 935–938.

25. Mazurkiewicz-Pisarek, A., Płucienniczak, G., Ciach, T., and Płucienniczak, A. (2016). The factor VIII protein and its function. Acta Biochim. Pol. *63*, 11–16.

26. Zhang, A.H., Yoon, J., Kim, Y.C., and Scott, D.W. (2018). Targeting antigen-specific B cells using antigen-expressing transduced regulatory T cells. J. Immunol. *201*, 1434–1441.

27. Parvathaneni, K., and Scott, D.W. (2018). Engineered FVIII-expressing cytotoxic T cells target and kill FVIII-specific B cells in vitro and in vivo. Blood Adv. *2*, 2332–2340.

28. Gangadharan, B., Ing, M., Delignat, S., Peyron, I., Teyssandier, M., Kaveri, S.V., and Lacroix-Desmazes, S. (2017). The C1 and C2 domains of blood coagulation factor VIII mediate its endocytosis by dendritic cells. Haematologica *102*, 271–281.

29. Peyron, I., Hartholt, R.B., Pedró-Cos, L., van Alphen, F., Brinke, A.T., Lardy, N., Meijer, A.B., and Voorberg, J. (2018). Comparative profiling of HLA-DR and HLA-DQ associated factor VIII peptides presented by monocyte-derived dendritic cells. Haematologica *103*, 172–178.

30. Steinitz, K.N., van Helden, P.M., Binder, B., Wraith, D.C., Unterthurner, S., Hermann, C., Schuster, M., Ahmad, R.U., Weiller, M., Lubich, C., et al. (2012). $CD4^+$ T-cell epitopes associated with antibody responses after intravenously and subcutaneously applied human FVIII in humanized hemophilic E17 HLA-DRB1*1501 mice. Blood *119*, 4073–4082.

31. Wright, J.F. (2009). Transient transfection methods for clinical adeno-associated viral vector production. Hum. Gene Ther. *20*, 698–706.

32. Wang, Q., Dong, B., Firrman, J., Roberts, S., Moore, A.R., Cao, W., Diao, Y., Kapranov, P., Xu, R., and Xiao, W. (2014). Efficient production of dual recombinant adeno-associated viral vectors for factor VIII delivery. Hum. Gene Ther. Methods *25*, 261–268.