SUPPLEMENTARY APPENDIX

N-linked glycosylation modulates the immunogenicity of recombinant human factor VIII in hemophilia A mice

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Supplementary Methods

Clearance of human rFVIII in mice

Mice were injected intravenously with 200 IU/kg of rFVIII via the tail vein. At the indicated timepoints, blood was collected from anesthetized mice via the retro-orbital plexus in one-tenth volume of 3.2% sodium citrate. Mice were sampled a maximum of 3 times from alternating retro-orbital plexes. Plasma was isolated by centrifugation. FVIII activity (FVIII:C) was measured using the Chromogenix Coatest SP4 Factor VIII (Diapharma, West Chester Township, OH) chromogenic kit using a standard curve generated from the corresponding rFVIII lot in FVIII-deficient pooled mouse plasma. Values were normalized to a sample collected 5 min after infusion.

Measurement of FVIII antigen and binding to murine VWF

FVIII antigen (FVIII:Ag) was measured by ELISA using a matched-pair of polyclonal FVIII antibodies according to the manufacturer's protocol (Affinity Biologicals, Ancaster, ON, Canada). Values were determined using a standard curve of CHO-rFVIII (1 IU/mL = 100 ng/mL).

Binding of rFVIII to murine VWF was measured by ELISA.[21] Maxisorp (Nunc, Roskilde, Denmark) microtitre plates were coated with a polyclonal rabbit anti-human VWF antibody (Dako, Glostrup, Denmark), which cross reacts with murine VWF, at 1 μg/mL overnight at 4°C. Murine VWF was captured from FVIII KO mouse pooled plasma over 2 hours. Serial dilutions of different rFVIII products were then incubated overnight. Bound rFVIII was detected with HRP-conjugated polyclonal sheep anti-human FVIII antibodies (Affinity Biologicals) and read at 492 nm.

FVIII deglycosylation

rFVIII was desialylated and de-N-glycosylated using α2-3,6,8 neuraminidase and PNGase F (New England Biolabs, Ipswich. MA), respectively, both under non-denaturing conditions at 37°C for 18 hours in the manufacturer's recommended buffer; controls were incubated in buffer without enzyme. The glycosidase was removed via dialysis (100 kDa MWCO) in PBS containing 0.5 μM Cu^{2+}. Glycan removal was confirmed by lectin binding assay. FVIII:C procoagulant activity and antigenicity was assessed by 1-stage clotting assay and FVIII:Ag ELISA (Affinity Biologicals), respectively.

IFN-γ ELISPOT

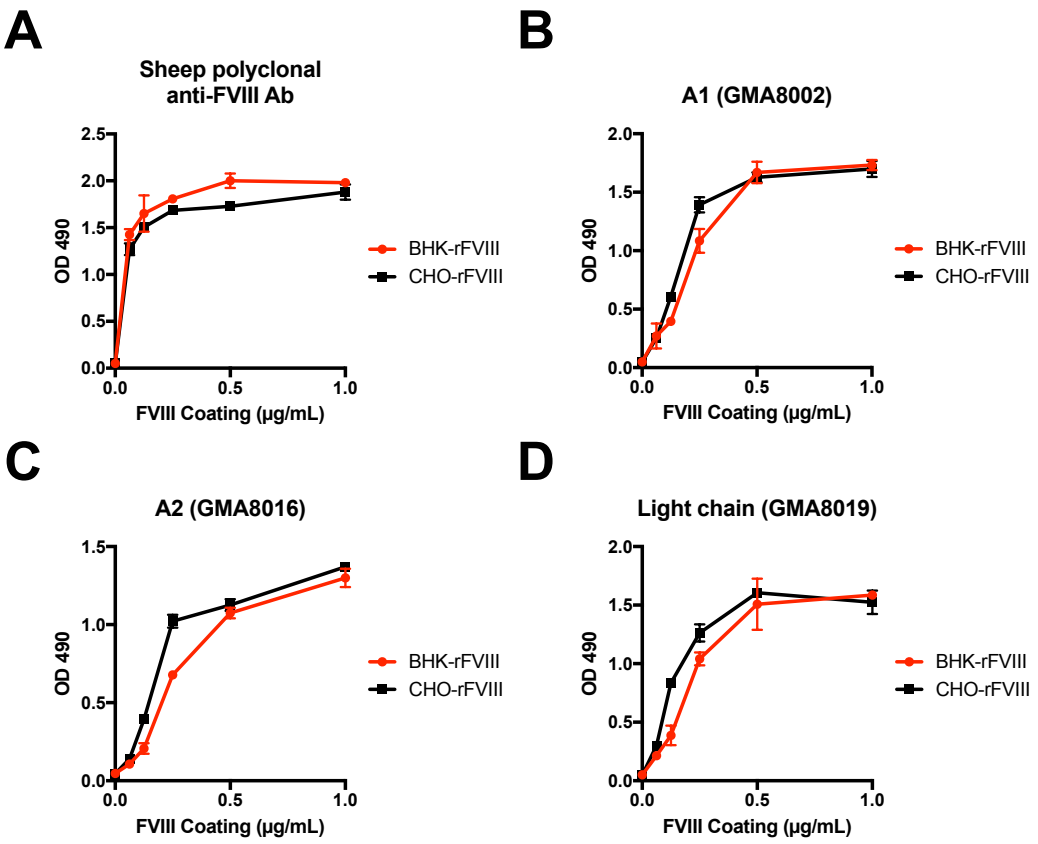
rFVIII-induced secretion of IFN-γ were quantified by ELISPOT according to the manufacturers protocol (Mouse IFN gamma ELISPOT Ready-SET-Go!, eBioscience). Splenocytes from naïve or rFVIII-primed mice were aseptically isolated and seeded into ELISPOT plate (EMD Millipore) at 5 x 10^5 cells/well in RPMI 1640 media supplemented with 10% FBS, 1% penicillin/streptomycin, 50 μM β-mercaptoethanol. Cells were then stimulated for 48 hours with different rFVIII conditions at 37°C . Total number of IFN-γ-secreting cells were calculated using an automated ELISpot counter (Cellular Technology). FVIII-specific spots were calculated by subtracting unstimulated wells.

In-vitro FVIII binding and uptake analyses

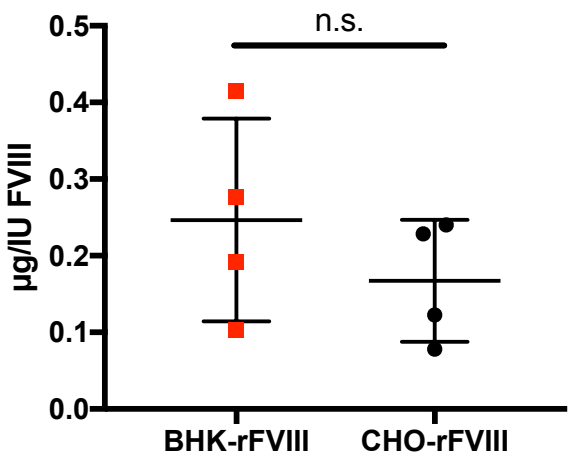
Splenocytes isolated from FVIII naïve hemophilia A mice were incubated with 0-1 µg/mL rFVIII or deglycosylated rFVIII at 4°C for 1 hour. Cells were stained using a FITC-conjugated polyclonal sheep anti-FVIII antibody (Affinity Biologicals), and anti-CD11c antibody (clone: N418, Invitrogen, Carlsbad, CA); the data were acquired on a MACSQuant Analyzer 10 (Miltenyi Biotec, Bergisch Gladbach, Germany) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Human samples

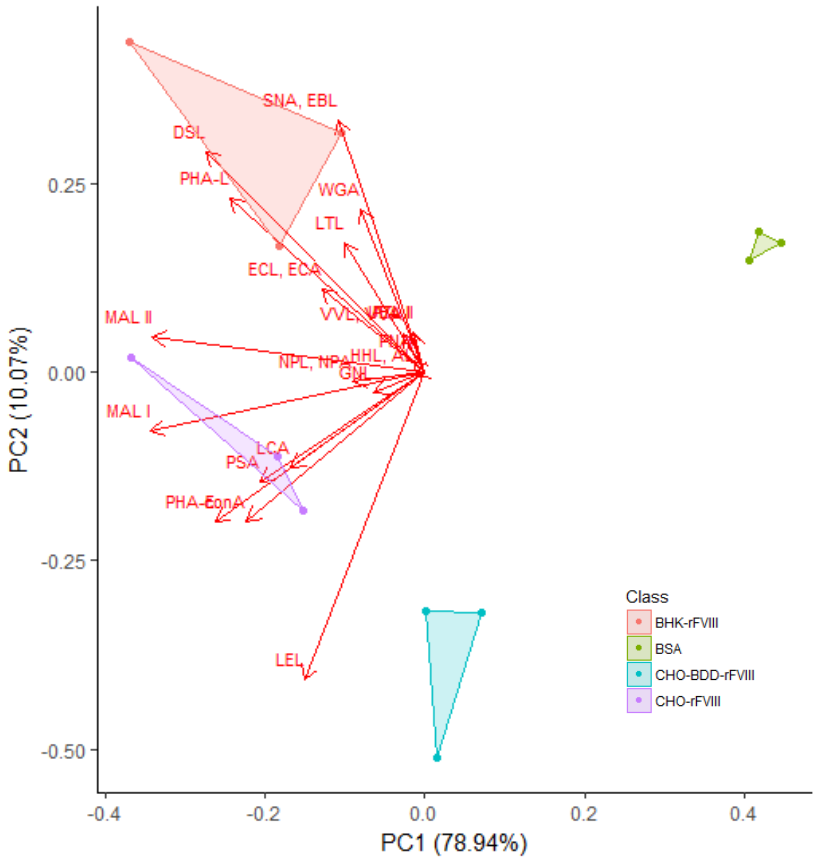
Plasma was isolated by centrifugation of whole blood collected from healthy male and female volunteers (age 22-65). All participants gave informed consent, and this study was approved by the Research Ethics Board of Queen's University, Kingston, Canada.



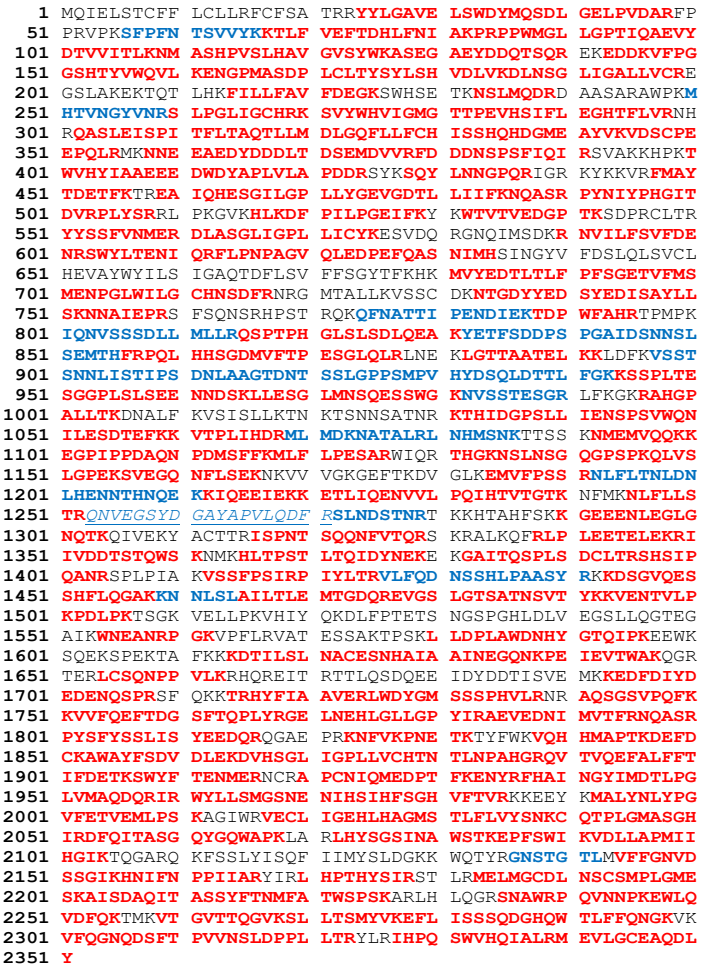
Supplementary Figure 1. Saturation of Nunc Maxisorp microtitre plates with BHK- and CHO-rFVIII. FVIII was adsorbed at varying concentrations between 0-1 µg/mL overnight at 4°C. The amount of FVIII adsorbed was detected using (A) a sheep anti-human FVIII polyclonal antibody, and mouse anti-human FVIII monoclonal antibodies directed against the (B) A1 domain, (C) A2 domain, and (D) light chain. Line and error bars represent mean and SD, respectively. Data is shown from 1 of 3 technical replicates.



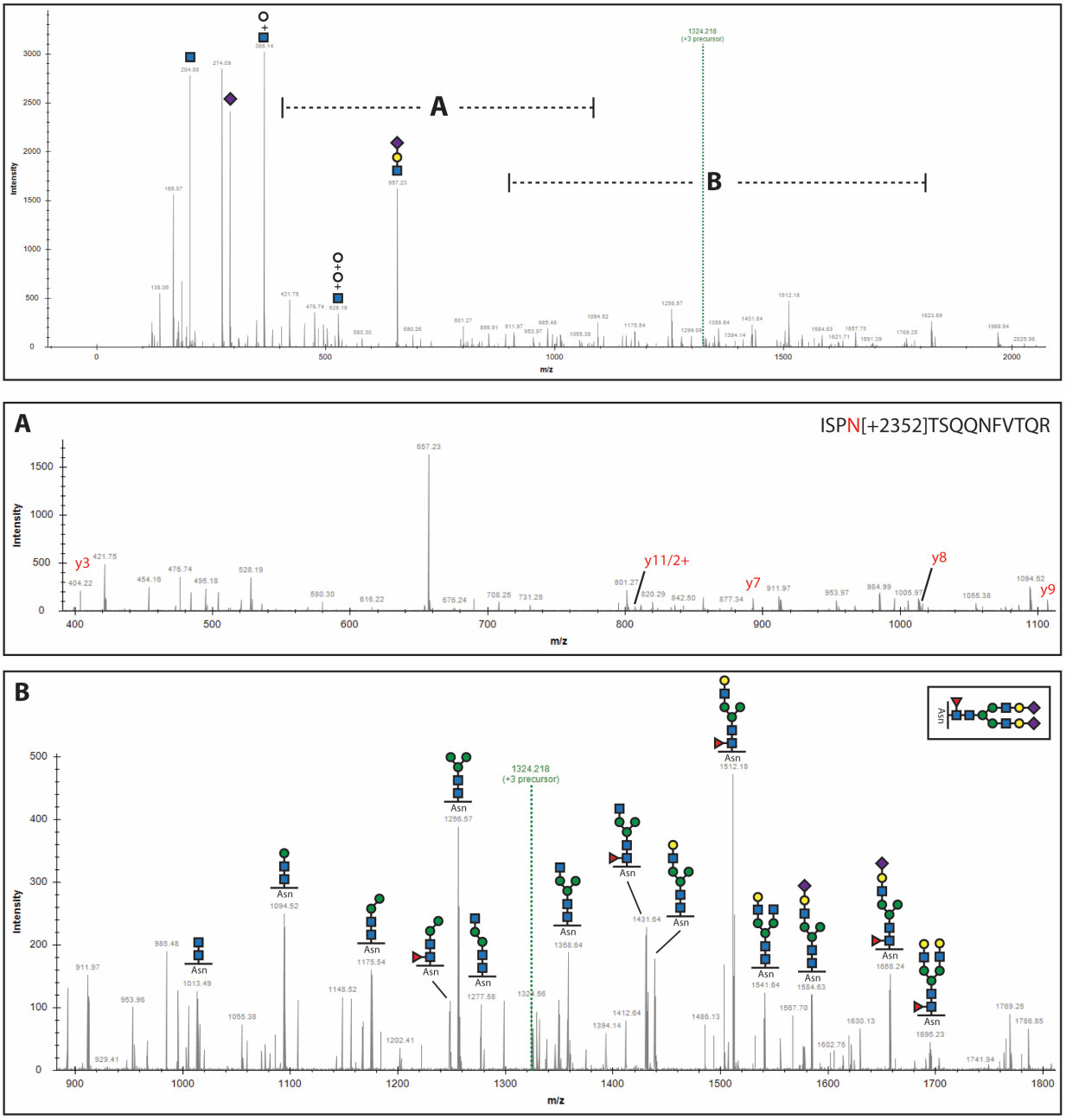
Supplementary Figure 2. Ratio of protein to FVIII activity in different lots of BHK- and CHO-rFVIII. Four distinct lots of rFVIII were reconstituted and total protein was assessed by Bradford assay against a bovine serum albumin standard curve. Data points are reported as µg of protein per IU of FVIII according to the manufacturer's label. Line and error bars represent mean and SD, respectively. Statistical significance was determined by Mann-Whitney U test.



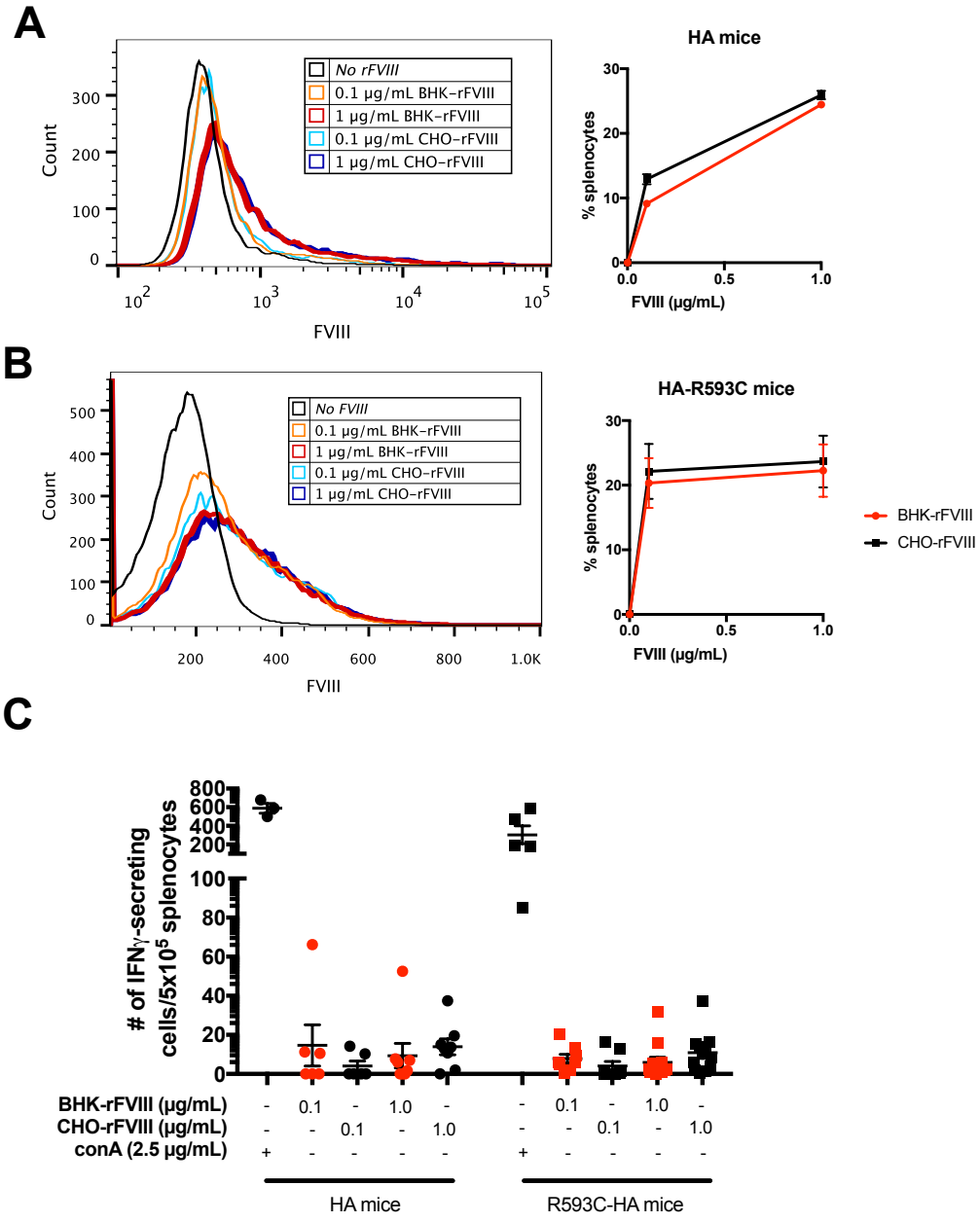
Supplementary Figure 3. Principal component analysis of the lectin binding dataset for rFVIII products. Data from 3 technical replicates of the lectin binding assay on 1 lot of each rFVIII product were analyzed by principal components using R and the ggplot2 package. PC1, the first principal component explains 78.94% of the variance; PC2, the second principal component explains 10.07% of variance. The red arrows represent the specific lectin that contribute to the identity of each cluster.



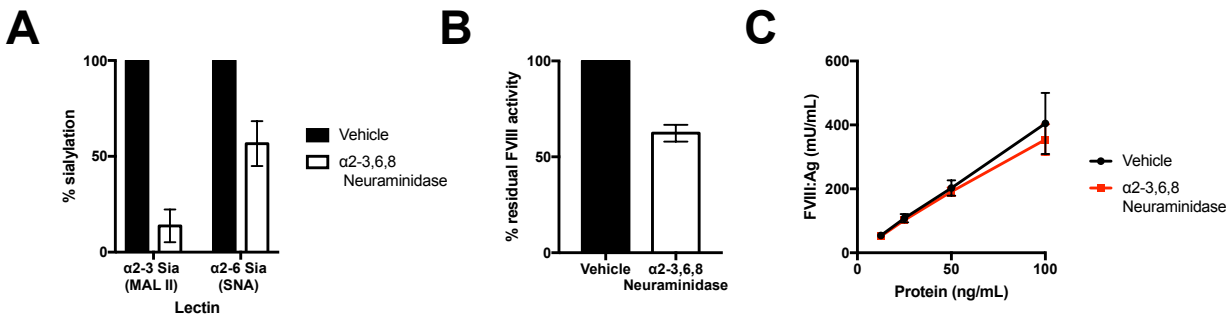
Supplementary Figure 4. Peptide coverage map. Using CHO-rFVIII as an example, Mascot search of the peptides detected by LC-MS/MS showed a 78.1% sequence coverage of full-length FVIII (19 amino acid signal peptide included here). Detected peptides are marked in red and the N-glycan consensus sequences, N_X_S/T, are marked in blue.



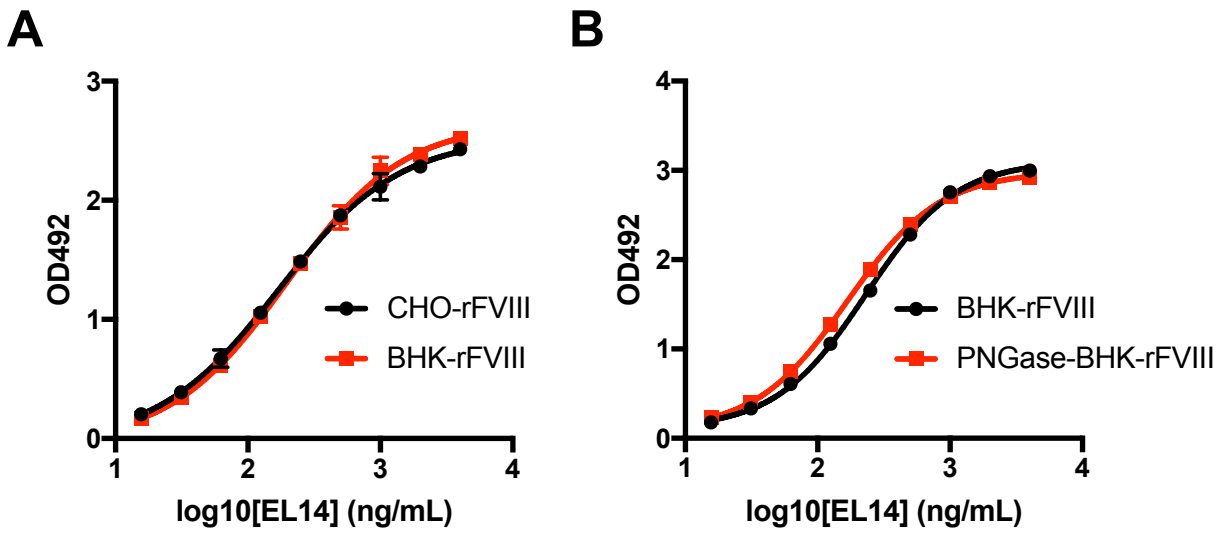
Supplementary Figure 5. Reconstruction of an N-linked glycan at Asn1300 on BHK-rFVIII. Y-ions are depicted in red. Glycan structures are depicted above each peak where blue squares, green circles, yellow circles, red triangles and purple diamonds represent N-acetylglucosamine, mannose, galactose, fucose and sialic acid, respectively. Open circles represent either mannose or galactose. Based on the mass of the peptide and the glycan, we can conclude that this is a bi-antennary fucosylated glycan attached to Asn1300.



Supplementary Figure 6. Different sources of full-length rFVIII do not elicit differences in binding to, or immune reactivity in naïve splenocytes. Splenocytes from C57Bl/6 HA mice (A) and transgenic R593C-FVIII mice (B) were incubated with increasing concentrations of either BHK-rFVIII or CHO-rFVIII. Binding was assessed by flow cytometry. (C) Naïve splenocytes from HA mice were stimulated with rFVIII concentrates and assessed for interferon (IFN)ɣ-secreting cells by ELISPOT. The background signal from untreated splenocytes was subtracted from each condition. Treatment with concanavalin A (2.5 µg/mL) was used as a positive control. (Line and error bars represent mean and SEM, respectively. Data representative of at least 3 biological replicates.



Supplementary Figure 7. Characterization of neuraminidase-treated rFVIII and *in vitro* interactions with splenocytes and dendritic cells. Terminal sialic acid was removed from BHK-rFVIII using α2-3,6,8 neuraminidase for 18 hr at 37°C. Incubation in the absence of the glycosidase served as a vehicle control. Samples were dialyzed for 12 hr in PBS supplemented for 5 μM Cu^{2+} to remove the enzyme, and assessed for (A) desialylation by lectin binding assay, (B) residual FVIII activity by one-stage clotting assay, as well as (C) FVIII antigenicity by ELISA. Line and error bars represent mean and SEM respectively. Data representative of at least 3 independent experiments.



Supplementary Figure 8. Binding curve of EL14 to CHO-rFVIII, BHK-rFVIII, and PNGase-BHK-rFVIII. FVIII products were adsorbed to microtitre plates at saturating concentrations and incubated with increasing concentrations of the recombinant monoclonal EL14 antibody for 2 hours. (A) Comparison between CHO-rFVIII and BHK-rFVIII. (B) Comparison between BHK-rFVIII and de-N-glycosylated BHK-rFVIII. Error bars represent SD. Data representative of at least 3 independent experiments.

Supplementary Table 1. Lectin binding statistics between rFVIII products.

		CHO-rFVIII vs BHK-rFVIII	CHO-rFVIII vs CHO-BDD-rFVIII	BHK-rFVIII vs CHO-BDD-rFVIII
Lectin	Specificity	*P* value	*P* value	*P* value
ConA	αMan, αGlc	0.764	0.700	0.979
PSA	αMan, αGlc	0.523	0.364	0.813
LCA	αMan, αGlc	0.707	0.658	0.967
GNL	αMan	**0.003**	**0.001**	**0.004**
HHL, AL	αMan	**0.020**	**0.014**	0.109
NPL, NPA	αMan	**0.005**	**0.001**	0.065
PTL II	GalNAc, Gal	**0.008**	0.261	**0.007**
VVL, VVA	GalNAc	0.077	**0.042**	**0.004**
PNA	Galβ3GalNAc	0.901	0.051	**0.016**
MAL I	Galβ3GlcNAc	0.598	**0.014**	**0.037**
ECL, ECA	Galβ4GlcNAc	0.349	**0.007**	**0.004**
DSL	(GlcNAc)2-4	0.756	0.082	0.057
LEL	(GlcNAc)2-4	0.096	0.976	0.287
WGA	GlcNAc, sialic acid	**4.11E-04**	**0.003**	**2.07E-05**
MAL II	Neu5Acα3GalβGalNAc	0.737	**0.020**	**0.010**
SNA, EBL	Neu5Acα6Gal/GalNAc	**0.021**	0.288	**0.014**
UEA I	αFuc	**3.91E-04**	0.283	**3.83E-04**
LTL	αFuc	**0.019**	0.065	**0.001**
PHA-E	Galβ4GlcNAcβ2Manα6(GlcNAcβ4)(GlcNAcβ4Manα3)Manβ4	0.678	0.620	0.989
PHA-L	Galβ4GlcNAcβ6(GlcNAcβ2Manα3)Manα3	0.605	**0.006**	**0.007**