

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

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ABSTRACT

Immune responses to factor VIII remain the greatest complication in the treatment of severe hemophilia A. Recent epidemiological evidence has highlighted that recombinant factor VIII produced in baby hamster kidney cells is more immunogenic than factor VIII produced in Chinese hamster ovary cells. Glycosylation differences have been hypothesized to influence the immunogenicity of these synthetic concentrates. In two hemophilia A mouse models, baby hamster kidney cell-derived factor VIII elicited a stronger immune response compared to Chinese hamster ovary cell-derived factor VIII. Furthermore, factor VIII produced in baby hamster kidney cells exhibited accelerated clearance from circulation independent of von Willebrand factor. Lectin and mass spectrometry analysis of total N-linked glycans revealed differences in high-mannose glycans, sialylation, and the occupancy of glycan sites. Factor VIII desialylation did not influence binding to murine splenocytes or dendritic cells, nor surface co-stimulatory molecule expression. We did, however, observe increased levels of immunoglobulin M specific to baby hamster kidney-derived factor VIII in naïve hemophilia A mice. De-N-glycosylation enhanced immunoglobulin M binding, suggesting that N-glycan occupancy masks epitopes. Elevated levels of immunoglobulin M and immunoglobulin G specific to baby hamster kidney-derived factor VIII were also observed in healthy individuals, and de-N-glycosylation increased immunoglobulin G binding. Collectively, our data suggest that factor VIII produced in baby hamster kidney cells is more immunogenic than that produced in Chinese hamster ovary cells, and that incomplete occupancy of N-linked glycosylation sites leads to the formation of immunoglobulin M- and immunoglobulin G-factor VIII immune complexes that contribute to the enhanced clearance and immunogenicity in these mouse models of hemophilia A.

Introduction

The immune response that develops in ~30% of severe hemophilia A (HA) patients remains the most serious complication in factor VIII (FVIII) replacement therapy. Why FVIII-neutralizing antibodies, known as inhibitors, develop in only some patients remains unclear. The type of FVIII concentrate has been proposed as one of the factors that influences the risk of FVIII immunogenicity. Three independent cohort studies have described differences among different recombinant (r) FVIII concentrates, where a 2nd generation full-length rFVIII was associated with a 1.6-fold increase in inhibitor risk compared to 3rd generation products.¹⁻³ Most recently, a fourth retrospective analysis further reported hazard ratios of 2.81 and 1.64 for inhibitor incidence with 2nd and 3rd generation rFVIII, respectively, compared to plasma-derived FVIII.⁴

While these observational studies provide compelling evidence, the mechanistic basis for such findings has only been hypothesized, and not systematically examined.⁵ The transition from 2nd to 3rd generation rFVIII is based on the removal of human and animal proteins in the production process and final product formulation.

Haematologica 2018
Volume 103(11):1925-1936

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Received: January 11, 2018.

Accepted: July 9, 2018.

Pre-published: July 12, 2018.

doi:10.3324/haematol.2018.188219

Check the online version for the most updated information on this article, online supplements, and information on authorship & disclosures: www.haematologica.org/content/103/11/1925

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In the aforementioned studies, 2nd and 3rd generation products refer specifically to Kogenate FS® (Bayer) produced in baby hamster kidney cells (BHK; BHK-rFVIII), and Advate® (Shire) produced in Chinese hamster ovary cells (CHO; CHO-rFVIII). In addition, these products differ by a single amino acid (AA) at position 1241 in the FVIII B domain, aspartic acid and glutamic acid, respectively. However, the immunologic relevance of this substitution appears insignificant as AA 1241 is poorly represented in both the major histocompatibility class II-restricted peptide of human monocyte-derived dendritic cells (DCs), and the repertoire of DR15-restricted CD4⁺ T-cell epitopes.^{6,7} Commercial formulations of BHK-rFVIII have very recently been reported to contain a higher presence of protein aggregates, which have previously been shown to be immunogenic.^{8,9}

Another hypothesis proposes that the differential immunogenicity is attributed to post-translational modification of rFVIII, and specifically to differential glycosylation patterns between BHK- and CHO-rFVIII at the 25 potential N-linked sites.^{5,10} Glycans have been implicated in FVIII intracellular trafficking and folding, as well as clearance by the asialoglycoprotein receptor and Siglec-5.¹¹⁻¹⁴ High-mannose glycans have been hypothesized to facilitate the uptake of FVIII *via* the mannose receptor on DCs and macrophages, however the data have been conflicting, and the *in vivo* significance of this interaction is unclear.^{15,16} Previous non-clinical studies have reported similar, or increased, immunogenicity of BHK-rFVIII compared to CHO-rFVIII, as well as a decreased, but statistically insignificant, inhibitory antibody response to deglycosylated FVIII.¹⁷⁻¹⁹ No mechanistic explanation for these differences has been provided.

Here, we used the previously described BHK- and CHO-rFVIII concentrates, Kogenate FS® and Advate®, respectively, and assessed their relative immunogenicities in two complementary murine models of HA. We further characterized the glycosylation profiles of each product, and evaluated their role in the development of the anti-FVIII immune response in these murine models.

Methods

FVIII concentrates

The following human rFVIII concentrates were used: Kogenate FS® (full-length BHK-rFVIII; Bayer, Leverkusen, Germany), Advate® (full-length CHO-rFVIII; Shire, Dublin, Ireland), and Xyntha® (CHO-B-domain deleted (BDD)-rFVIII; Pfizer, New York City, NY, USA). Information on FVIII clearance, antigen/activity assays, deglycosylation, and von Willebrand factor (VWF) binding is available in the *Online Supplementary Methods*.

Mice

Sex and littermate-matched 8-12 week old C57Bl/6 *F8* exon 16 knockout (KO) mice with a human full-length *F8* transgene containing an R593C point mutation (HA-R593C mice) that is transcribed, but for which FVIII protein is undetectable in plasma, were used for preliminary experiments.²⁰ Results were extended using similarly controlled “conventional” C57Bl/6 *F8* exon 16 KO mice (HA mice).²¹ Mice were treated by subcutaneous or tail vein intravenous injection of 6 IU (240 IU/kg; as per manufacturer’s label) of rFVIII biweekly for two weeks. Lipopolysaccharide (LPS; 1 µg) was used as an adjuvant with the first FVIII infusion where indicated. HA mice were challenged with 2 IU (80 IU/kg) of rFVIII

using the same regimen. Blood was collected by cardiac puncture in one-tenth volume of 3.2% sodium citrate 28 days after the first administration of FVIII. Mouse experiments were approved by the Queen’s University Animal Care Committee.

Anti-FVIII antibodies and FVIII inhibitor assays

FVIII-specific immunoglobulin G (IgG) titres were quantified by enzyme-linked immunosorbent assay (ELISA) and FVIII inhibitors were measured by a 1-stage FVIII clotting assay using an automated coagulometer (Siemens BCS XP, Berlin, Germany), as previously described.^{22,23} Where indicated, anti-FVIII IgG was quantified using a standard curve generated using the human anti-FVIII monoclonal antibody, EL14 (provided by Dr. Jan Voorberg, Sanquin Research, Amsterdam, The Netherlands).²⁴ Information on human sample collection is available in the *Online Supplementary Methods*.

FVIII-specific IgM was assessed by indirect ELISA. rFVIII (1 µg/mL) was adsorbed to Nunc Maxisorp 96-well plates overnight. Samples were diluted 1:20 and incubated for 2 hrs. IgM was detected using horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-human IgM (Southern Biotech, Birmingham, AL, USA). Bovine serum albumin (BSA)-coated wells were used as controls. Plates were developed for 15 minutes using o-phenylenediamine (Sigma, St. Louis, MO, USA) and read at 492 nm.

Lectin binding assays

rFVIII products were adsorbed to Maxisorp microtitre plates at 1 µg/mL overnight at 4°C. All products saturated binding at this concentration (*Online Supplementary Figure S1*). Plates were blocked with 1% BSA in phosphate buffered saline (PBS) + 0.01% Tween-20 for 1 hr, and subsequently incubated with biotinylated lectins (Vector Laboratories, Burlingame, CA, USA) for 30 min. Detection was facilitated using streptavidin-poly-HRP (ThermoFisher Scientific, Waltham, MA, USA) and developed for 5 min. Statistical analysis was performed using the Student *t* test.

FVIII preparation for mass spectrometry

FVIII samples were desalted on ViVaspin, 50kDa MWCO (Sartorius, Goettingen, Germany) spin columns. 20 µg of protein in 400 µl of 50 mM ammonium bicarbonate was reduced in 10 mM dithiothreitol (DTT) at 60°C for 40 min then alkylated in 25 mM iodoacetamide in darkness for 30 min. The reaction was stopped by the addition of 20 mM DTT in darkness for 40 min. Trypsin at 1:50 ratio was added to the sample and incubated at 37°C overnight.

Glycopeptide analysis by liquid chromatography and tandem mass spectrometry (LC-MS/MS)

Peptides were applied to a nano-HPLC Chip using an Agilent 1200 series microwell-plate autosampler interfaced with a Agilent 6550 Q-TOF MS (Agilent Technologies, Santa Clara, CA, USA). The reverse-phase nano-HPLC Chip (G4240-62002) had a 40 nL enrichment column and a 75 µm x 150 mm separation column packed with 5 µm Zorbax 300SB-C18. The mobile phase was 0.1% formic acid in water (v/v) as solvent A, and 0.1% formic acid in ACN (v/v) as solvent B. The flow rate was 0.3 µL/min with gradient schedule; 3% B (0-1 min); 3-40% B (1-90 min); 40-80% B (90-95 min); 80% B (95-100 min) and 80-3% B (100-105 min).

Mascot search was used to identify proteins and sequence coverage. Extracted glycopeptides were identified by Agilent Masshunter Quantitative Analysis software by the presence of hexose and N-acetylhexosamine. Glycan structures were predicted for extracted glycopeptides by GlycoMod. Glycan structure by MS/MS and occupancy of consensus N_XS/T N-glycosylation sites were determined manually.

Results

BHK-rFVIII is more immunogenic than CHO-rFVIII in mouse models of hemophilia A

While an International Standard exists for determination of the FVIII:C potency of FVIII concentrates (and products are labeled in International Units), there is no standardized method to accurately quantify FVIII:Ag between products.²⁵ Although there are differences in specific coagulant activity between commercial preparations (and between different lots of concentrate) (*Online Supplementary Figure S2*), we dosed rFVIII by the coagulant activity reported by the manufacturer so as to recapitulate clinical practice and the data reported by previous FVIII inhibitor epidemiological studies. To assess the immunogenic differences between CHO- and BHK-rFVIII, we administered rFVIII subcutaneously or intravenously to HA-R593C mice biweekly (6 IUs; 240 IU/kg per administration) for two weeks and analyzed plasma on day 28 (Figure 1A). After subcutaneous immunization, we observed a significant increase in the incidence of anti-FVIII IgG antibodies in mice treated with BHK-rFVIII compared to CHO-rFVIII 28 days after the initial injection (93.75% vs. 47.3% respectively, $P=0.0042$; Figure 1B). Further serological analysis showed that the α FVIII IgG titre was also greater when mice were immunized with BHK-rFVIII (Figure 1C). A similar difference was observed in the incidence of all FVIII inhibitory antibodies (93.75% vs. 36.8%, $P=0.0003$; Figure 1D). However no significant differences were observed in the inhibitory antibody titres (Figure 1E).

To account for the typical biodistribution of FVIII in the bloodstream, we administered rFVIII intravenously with the first injection containing 1 μ g of lipopolysaccharide (LPS) as an adjuvant (Figure 1F). We did not observe any significant difference in the incidence of α FVIII IgG between products in these experiments (Figure 1G). However, BHK-rFVIII elicited higher titres of α FVIII IgG (Figure 1H) among FVIII-responders. Analysis of FVIII inhibitory antibodies showed no differences in incidence (Figure 1I) or inhibitor titre (Figure 1J) using this testing protocol.

All mice developed FVIII-specific antibodies by day 28 (Figure 2B), and there were no differences in the titre of FVIII-specific antibodies (Figure 2C). However, mice immunized with BHK-rFVIII exhibited a higher incidence of all FVIII antibodies with an inhibitory titer >1 Bethesda unit (BU; 100% vs. 54.5%; Figure 2D), but no difference in the magnitude among FVIII-responders (Figure 2E).

BHK-rFVIII exhibits accelerated clearance that is independent of binding to murine VWF

We next assessed the clearance of rFVIII from the mouse circulation. Following an intravenous infusion of either BHK-rFVIII or CHO-rFVIII at 200 IU/kg in HA mice, plasma FVIII:C was measured by chromogenic assay at the indicated time points and normalized to a 5 min post-infusion sample. Our results show a significant increase in the clearance rate of BHK-rFVIII compared to CHO-rFVIII (6.22 hrs vs. 9.53 hrs; $P=0.02$) (Figure 3A).

Given the dominant influence of VWF on FVIII half-life, we assessed whether the rFVIII products exhibit differential binding to endogenous mouse VWF (Figure 3B). FVIII-binding was reported as a function of the amount of FVIII:Ag at each dilution in the assay. These data suggest

that there is no significant difference in the binding to murine VWF between CHO- and BHK-derived rFVIII, and that the differences observed in clearance and immunogenicity are independent of VWF and may suggest structural moieties, such as post-translational modifications, that facilitate or inhibit cellular uptake.

rFVIII produced in BHK or CHO cell lines contains significantly different glycosylation profiles

Given the differences in clearance and immunogenicity observed *in vivo*, we next assessed rFVIII glycosylation by using a panel of lectins to detect specific, exposed, carbohydrate linkages on CHO- and BHK-rFVIII. CHO-BDD-rFVIII was used as a control, as it lacks all but six of the potential N-linked sites. Multiple lectins specific for similar structures were used to confirm findings, and the median-centered optical absorbance readings were compared between products using the Student *t* test (*Online Supplementary Table S1*). These data suggest that BHK-rFVIII has a higher degree of sialylation and fucosylation, and a lower presence of high-mannose glycans compared to CHO-rFVIII (Figure 4A; Principal component analysis *Online Supplementary Figure S3*). We further confirmed that these glycan differences were conserved across three different lots of rFVIII products (Figure 4B).

Table 1. N-linked glycan structures detected across BHK-, CHO-, and CHO-BDD-rFVIII products.

Name	Abbreviation	Structure
(Hex)2 +(Man)3(GlcNAc)2	H2	
(Hex)3 +(Man)3(GlcNAc)2	H3	
(Hex)4 +(Man)3(GlcNAc)2	H4	
(Hex)5 +(Man)3(GlcNAc)2	H5	
(Hex)6 +(Man)3(GlcNAc)2	H6	
(Hex)1(HexNAc)1 +(Man)3(GlcNAc)2	H1N1	
(Hex)2(HexNAc)2 +(Man)3(GlcNAc)2	H2N2	
(Hex)2(HexNAc)2(Fuc)1 +(Man)3(GlcNAc)2	H2N2F1	
(Hex)3(HexNAc)1 +(Man)3(GlcNAc)2	H3N1	
(Hex)3(HexNAc)3(Fuc)1 +(Man)3(GlcNAc)2	H3N3F1	
(Hex)2(HexNAc)1(NeuAc)1 +(Man)3(GlcNAc)2	H2N1S1	
(Hex)2(HexNAc)2(NeuAc)1 +(Man)3(GlcNAc)2	H2N2S1	
(Hex)2(HexNAc)2(Fuc)1(NeuAc)1 +(Man)3(GlcNAc)2	H2N2F1S1	
Hex(2)(HexNAc)2(Fuc)2(NeuAc)1 +(Man)3(GlcNAc)2	H2N2F2S1	
(Hex)3(HexNAc)1(NeuAc)1 +(Man)3(GlcNAc)2	H3N1S1	
(Hex)3(HexNAc)3(Fuc)1(NeuAc)1 +(Man)3(GlcNAc)2	H3N3F1S1	
(Hex)3(HexNAc)3(Fuc)1(NeuAc)2 +(Man)3(GlcNAc)2	H3N3F1S2	
(Hex)2(HexNAc)2(NeuAc)2 +(Man)3(GlcNAc)2	H2N2S2	
(Hex)2(HexNAc)2(Fuc)1(NeuAc)2 +(Man)3(GlcNAc)2	H2N2F1S2	
(Hex)3(HexNAc)3(Fuc)1(NeuAc)3 +(Man)3(GlcNAc)2	H3N3F1S3	
(Hex)3(HexNAc)3(Fuc)1(NeuAc)4 +(Man)3(GlcNAc)2	H3N3F1S4	

By LC-MS/MS, we detected 22 of the 25 potential N-linked Asn-X-Ser/Thr consensus sequences, and identified a total of 21 unique glycans (Table 1; for peptide coverage and glycan construction see *Online Supplementary Figure S4* and *Figure S5*). The intensity of glyco-peptides was normalized to the area under the curve of non-glycosylated peptides relative to CHO-rFVIII. The predicted N-linked

glycan sites at Asn1001, Asn1005, and Asn1512 were not detected by our methods. At each occupied site, we observed significant heterogeneity in the glycan structures (Figure 5A). We next grouped glycan structures into either high-mannose, asialylated, partially sialylated, or fully sialylated. In agreement with our lectin binding data, we observed high-mannose glycans at Asn757 and Asn1300

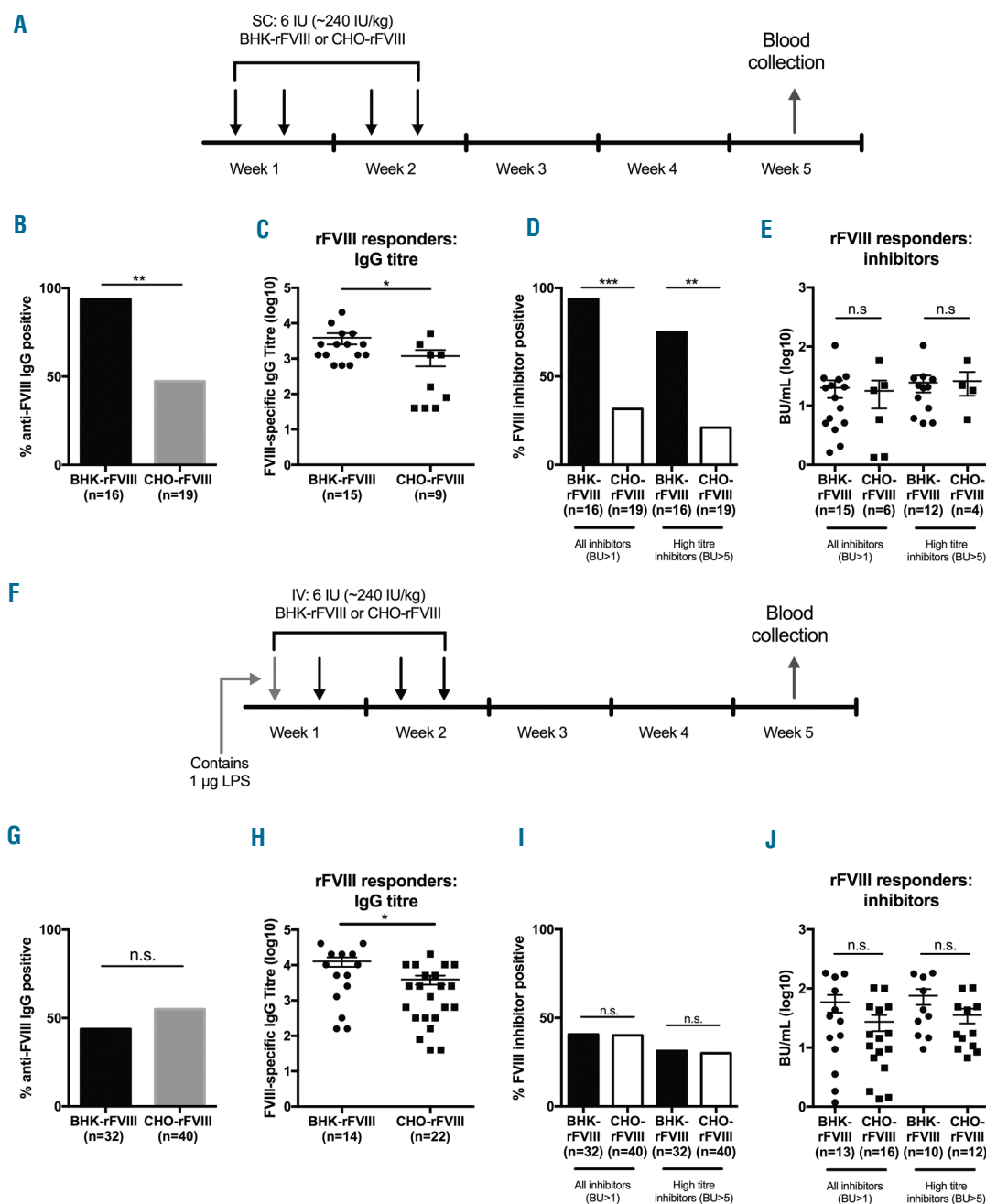


Figure 1. Immunogenic differences between BHK-rFVIII and CHO-rFVIII in HA-R593C mice. (A) Mice were immunized subcutaneously (SC) with 6 IU (0.6 µg; 240 IU/kg) of either BHK- or CHO-rFVIII biweekly for 2 weeks. Blood was collected by cardiac puncture 28 days after the first infusion. Plasma was assessed for (B) the incidence and (C) titre of FVIII-specific IgG, as well as (D) the incidence of inhibitors above 1 and 5 BU and (E) the magnitude of inhibitory activity. (F) Mice were immunized intravenously (IV) with 6 IU (0.6 µg; 240 IU/kg) of either BHK- or CHO-rFVIII product twice weekly for 2 weeks. The first infusion contained 1 µg of lipopolysaccharide. Plasma samples 28 days after the first infusion were assessed for (G) the incidence and (H) titre of FVIII-specific IgG. (I) Comparison of the incidence of inhibitors above 1 BU and the (J) magnitude of inhibitory activity. Horizontal lines and error bars represent the mean and SEM, respectively. Mann-Whitney U and Fisher's exact test were used where appropriate. **P*<0.05; ***P*<0.01; ****P*<0.001. BHK: baby hamster kidney cells; CHO: Chinese hamster ovary cells; rFVIII: recombinant factor VIII; Ig: immunoglobulin; n.s. not significant; BU: Bethesda unit.

in CHO-rFVIII but not in BHK-rFVIII (Figure 5B). Similarly, the sialylation of sub-terminal galactose residues is more complete in BHK-rFVIII as evidenced by a high proportion of fully-sialylated glycans (Figure 5C-E). We did not observe differences in the frequency of fucosylated N-linked glycans between products (*data not shown*).

Glycosylation of rFVIII does not influence binding to or induction of IFN γ production by splenocytes or splenic dendritic cells *in vitro*

Glycosylation differences between CHO- and BHK-rFVIII may alter the association of FVIII with different cell types or receptors. To address this, we employed a FVIII binding assay to unsorted splenocytes from rFVIII naïve HA or HA-R593C mice. However, we did not observe significant differences in the binding of the different rFVIII preparations to these cells (*Online Supplementary Figure S6A,B*). We further assessed downstream immune responses to rFVIII in naïve mixed lymphocyte populations using an interferon (IFN) γ enzyme-linked immunospot (ELISPOT). Splenocytes were isolated from naïve HA mice or HA-R593C mice and stimulated with the two rFVIII proteins for 48 hr. The number of cells secreting IFN γ did not differ between the two rFVIII concentrates (*Online Supplementary Figure S6C*). Since these data suggest that the early rFVIII innate immune responses are similar between HA mice and HA-R593C mice, we used HA-R593C mice for subsequent experiments.

Differential FVIII immunogenicity in hemophilia A mouse models is not explained by differences in sialic acid content

Given that the degree of N-linked sialylation was the greatest source of glycan variation between BHK- and

CHO-rFVIII, we removed the terminal sialic acids from BHK-rFVIII using α 2-3,6,8 neuraminidase for 18 hr at 37°C. Desialylation was confirmed by lectin binding assay (*Online Supplementary Figure S7A*). Desialylated FVIII:C procoagulant function was 62.3% of the control, however, FVIII antigenicity was conserved (*Online Supplementary Figure S7B,C*). The removal of sialic acid from BHK-rFVIII did not influence the binding to splenocytes or CD11c⁺ dendritic cells (Figure 6A,B). Considering the potential immunomodulatory role of sialic acid, we next investigated whether different glycoforms of rFVIII could influence the expression of the co-stimulatory molecules, CD80 and CD86, on naïve or LPS-stimulated splenocytes and DCs. While we observed significant upregulation of both CD80 and CD86 in LPS-stimulated conditions, the removal of sialic acid did not influence surface co-stimulatory molecule expression in either splenocytes or DCs (Figure 6C, D). Similarly, HA-R593C mice treated subcutaneously with BHK-rFVIII or its desialylated glycoform, as described above, did not exhibit significant differences in the incidence or the titre of FVIII-specific IgG (Figure 6E, F).

N-linked glycans prevent binding of non-neutralizing IgM and IgG to rFVIII

We next evaluated the interaction of non-neutralizing IgM and IgG on rFVIII, and the ability of N-linked glycans to regulate this interaction. We found that incubation of naïve HA, or HA-R593C mouse plasma on a FVIII-coated microtitre plate resulted in the increased binding of BHK-rFVIII-specific IgM (Figure 7A,B) relative to CHO-rFVIII. FVIII-specific IgG was not detected (*data not shown*). Of note, competition through preincubation with other forms of FVIII showed that these IgM molecules are spe-

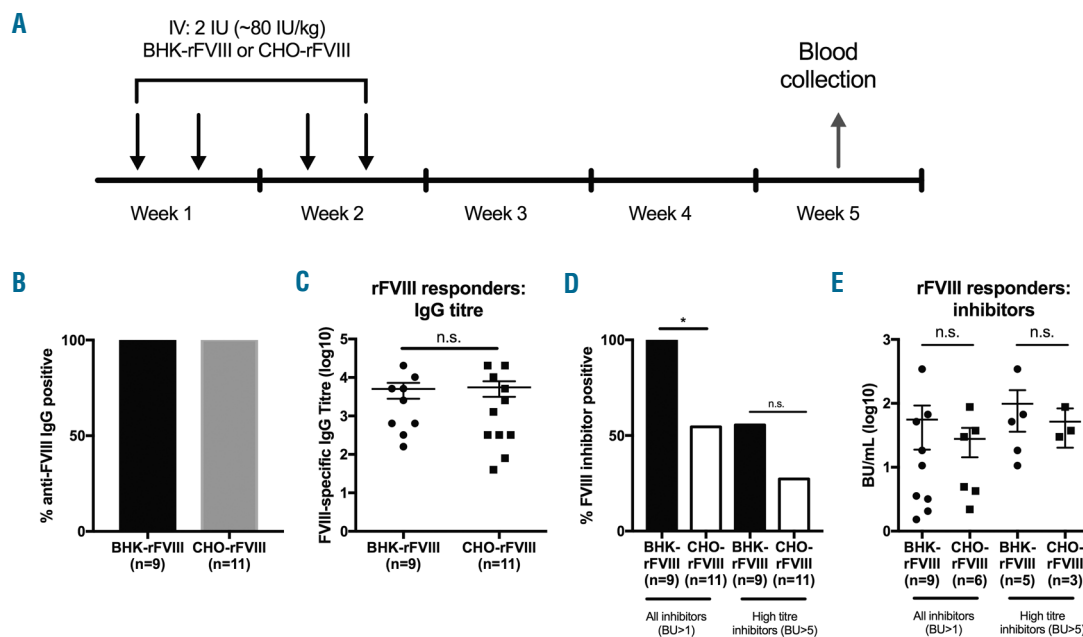


Figure 2. Immunogenic differences between BHK- and CHO-rFVIII in HA mice. (A) F8 exon 16 KO HA mice were immunized intravenously (IV) with 6 IU (0.6 μ g; 240 IU/kg) of either BHK- or CHO-rFVIII biweekly for 2 weeks. Blood was collected by cardiac puncture 28 days after the first infusion, and plasma was isolated by centrifugation. Samples were assessed for (B) incidence and (C) titres of FVIII-specific IgG. FVIII-responders were analyzed for (D) the incidence ($P=0.015$) and (E) concentration of FVIII inhibitors. The horizontal lines and error bars represent the mean and SEM. Mann-Whitney U test and Fisher's exact test were used where appropriate. * $P<0.05$. BHK: baby hamster kidney cells; CHO: Chinese hamster ovary cells; rFVIII: recombinant factor VIII; Ig: immunoglobulin; n.s. not significant; BU: Bethesda unit.

cific to BHK-rFVIII, and that the interaction is not inhibited by human VWF (Figure 7C).

We next evaluated whether N-linked glycans sterically hinder access of IgM to certain epitopes in the FVIII protein backbone. From our MS data, we observed that there were consistently higher proportions of unoccupied N-glycan sites at Asn900, Asn1255, Asn1259, Asn1282, Asn1300, and Asn1810 in BHK-rFVIII compared to CHO-rFVIII (Figure 7D). We subsequently removed all the N-linked glycans from BHK- and CHO-rFVIII using Peptide: N-Glycosidase F (PNGase F), and observed a greater increase in IgM binding to deglycosylated CHO-rFVIII (Figure 7E) when compared to the native rFVIII glycoform.

To extend these findings to humans, we collected plasma from healthy human volunteers and quantified levels of FVIII-specific IgM. We observed an increased binding of IgM to BHK-rFVIII compared to CHO-rFVIII and CHO-BDD-rFVIII (Figure 7F). The presence of FVIII-specific IgG antibodies has previously been reported in healthy individuals.^{26,27} We generated a standard curve using the recombinant antibody, EL14, which bound equally to rFVIII products (*Online Supplementary Figure S8*). Upon incubation with plasma from healthy human subjects, we observed that a higher proportion of human IgG bound to BHK-rFVIII compared to CHO-rFVIII (Figure 7G). We further determined that this binding was enhanced when the N-glycans were removed from rFVIII (Figure 7H).

Discussion

Factor VIII immunogenicity remains a significant concern among hemophilia treaters, and mounting evidence suggests that rFVIII products differ from pdFVIII concentrates as well as among themselves. Non-human cell lines have been shown to add immunogenic non-human glycan structures, Gal(α 1-3)Gal β 1-GlcNAc-R (α Gal) and 5-glycolylneuraminic acid (Neu5Gc) to rFVIII.^{28,29} In two murine models of HA, in which both non-human glycans are present, we found that BHK-rFVIII was more immunogenic than CHO-rFVIII. These data suggest that although Neu5Gc and α Gal have the potential to induce FVIII immunity in humans, as seen with the immune responses against cetuximab, there are additional mechanisms that contribute to this response.^{30,31}

As per its routine clinical use, mice were dosed by the procoagulant activity of rFVIII. However, the level of inactive FVIII protein in the commercial concentrates likely plays a role in immunogenicity. BHK-rFVIII has been reported to have higher FVIII:C and FVIII:Ag than advertised.³² We were unable to observe a similar trend across four lots of rFVIII, perhaps due to different methodologies used. High-dose intensive FVIII treatment has been implicated as a risk factor for inhibitor development, however this correlation is likely facilitated by the inflammatory milieu of concurrent surgery or bleeding.³³ Whether expo-

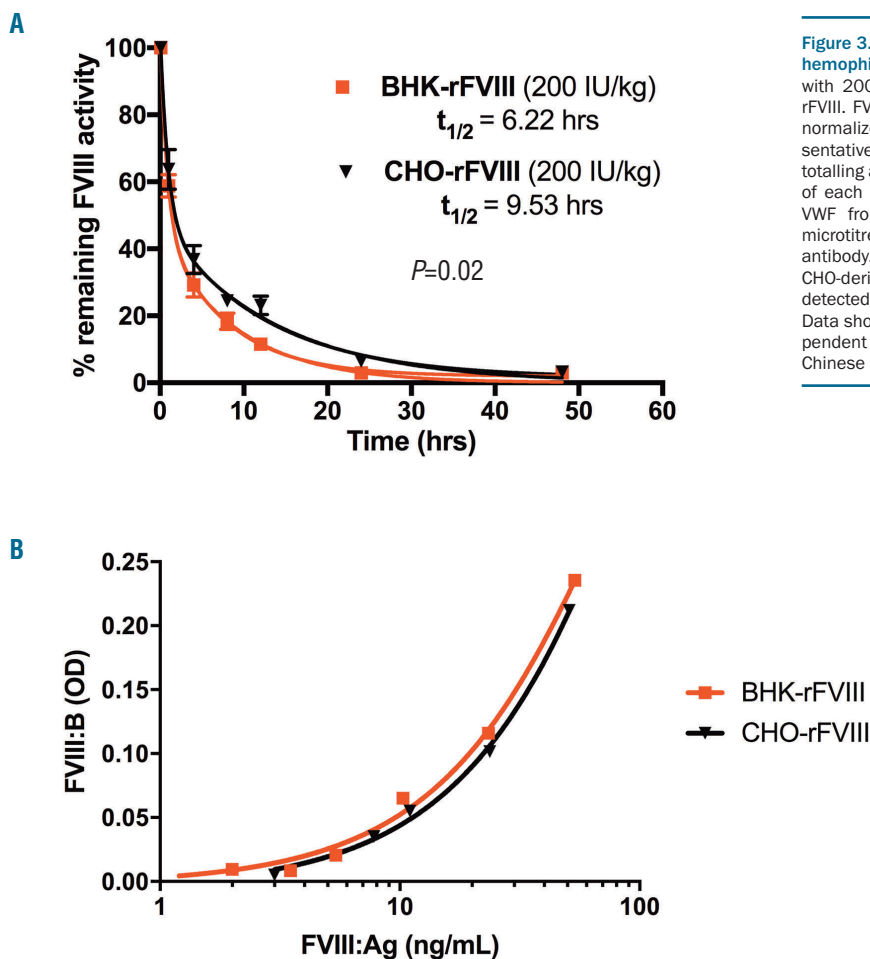


Figure 3. Characterization of FVIII half-life and VWF binding in hemophilia A mice. (A) HA mice were intravenously infused with 200 IU/kg of either BHK-rFVIII or CHO cell line-derived rFVIII. FVIII activity was measured by chromogenic assay, and normalized to a 5 min time point post-injection. Data are representative of the aggregate data of 3 independent experiments totalling at least 5 biological replicates per time point and 2 lots of each rFVIII product. Error bars represent SEM. (B) Murine VWF from HA mouse pooled plasma was captured on a microtitre plate coated with a polyclonal rabbit anti-human VWF antibody. Increasing concentrations of either BHK-derived, or CHO-derived rFVIII were incubated with the captured VWF and detected using a polyclonal sheep anti-human FVIII antibody. Data shown is one technical replicate representative of 3 independent experiments. BHK: baby hamster kidney cells; CHO: Chinese hamster ovary cells; rFVIII: recombinant factor VIII.

sure to greater amounts of FVIII protein accounts for the increase in immunogenicity in a non-inflammatory steady state is unclear. Consistent with our data, Delignat *et al.* standardized FVIII:Ag between products using human plasma, and observed enhanced immunogenicity of BHK-rFVIII compared to CHO-rFVIII in HA mice.¹⁸

The immunogenic disparity between rFVIII concentrates was greatest when administered subcutaneously in HA-R593C mice. In *F8* exon 16 KO HA mice, central tolerance for human FVIII is limited, which may explain why differences between very similar proteins are not as apparent in this mouse model.³⁴ In humanized *F8* exon 16 KO HA-R593C mice, immunological tolerance to human FVIII necessitates an adjuvant to elicit an immune response *via* intravenous administration, which may result in a general heightened immune reactivity against all antigens, thus preventing the resolution of subtle immunogenic differences.²⁰ Of note, there are major differences in the biodistribution of FVIII when administered intravenously, where it complexes with murine VWF and predominantly localizes in the liver and spleen, *versus* subcutaneous delivery, where VWF association is less likely, and FVIII localizes to the draining lymph node.³⁵ This likely directs FVIII to different populations of phagocytic cells, particularly DCs and macrophages.³⁶

The removal of FVIII from circulation can lead to clearance and/or antigen presentation. Even in the dominating

presence of endogenous murine VWF, BHK-rFVIII was cleared at an accelerated rate, independent of differences in VWF-binding under static conditions. This difference in half-life has not been described in human patients, and may only be apparent in this mouse model due to interspecies differences in the mechanisms of FVIII and VWF clearance. In this scenario, assuming that FVIII does not influence VWF clearance to a significant extent, it is possible that the ~5% of BHK-rFVIII that circulates without VWF is cleared faster, thus shifting the equilibrium to promote further FVIII dissociation from VWF.³⁷ We hypothesized that post-translational structures could contribute to the different clearance kinetics of these proteins in mice.

Our analysis of rFVIII glycans confirms the findings of previous studies: that rFVIII possesses predominantly core-fucosylated biantennary complex glycans, a heavily sialylated B domain, high mannose glycans at Asn239 and Asn2118, and unoccupied sites at Asn582, Asn943, Asn1384, and Asn1685.^{28,38,39} Contrary to previous studies, we did not detect tetraantennary glycans or Neu5Gc glycans (α Gal cannot be detected directly using our methods). Lectin binding analysis of exposed N- and O-linked glycans and mass spectrometry analysis of total N-linked glycans collectively showed differences in sialic acid and high-mannose glycan content. A similar analysis showed an absence of high mannose glycans as well as greater sialylation in CHO-derived rFVII compared to that produced

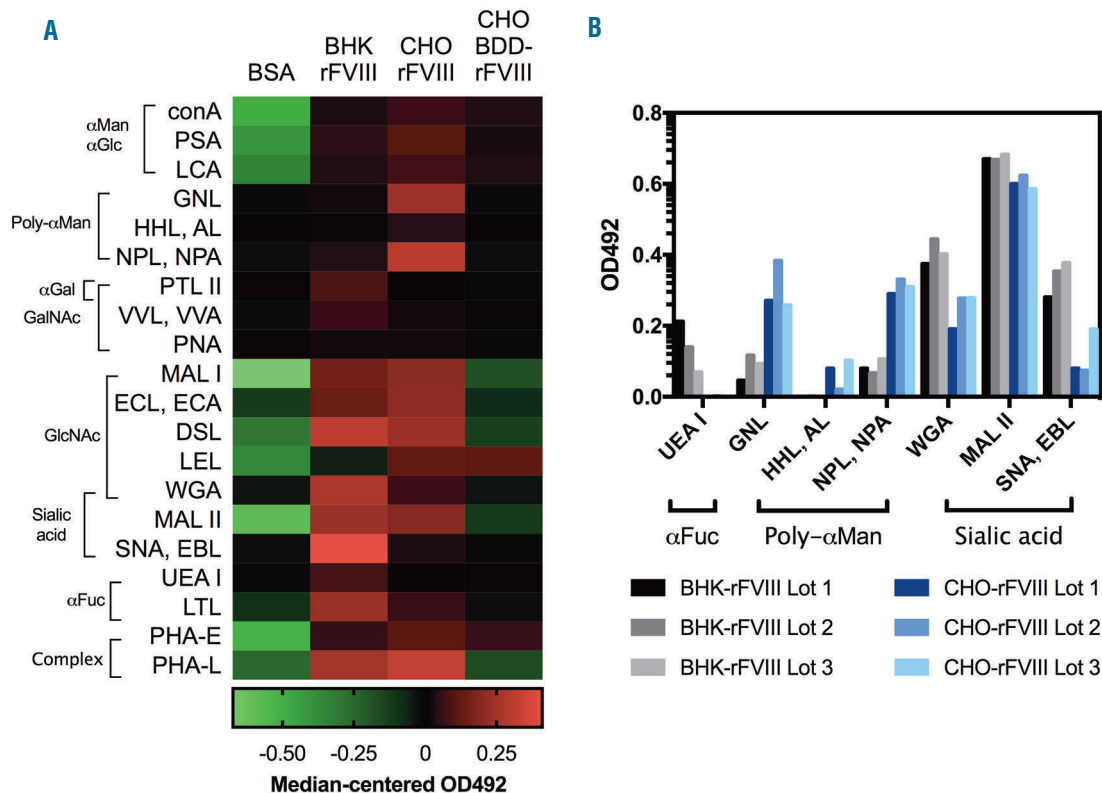


Figure 4. Lectin array analysis of exposed glycans on rFVIII products. rFVIII products were adsorbed on microtitre plates at 1 μ g/mL and assessed using a panel of biotinylated lectins. BSA was adsorbed as a control. Binding was detected using a streptavidin poly-HRP and read at 492 nm. (A) Heat plot demonstrating the median-centred binding values of lectins with varying carbohydrate specificities to different rFVIII products. Values shown are representative of the mean of at least 3 independent experiments on a single lot of each rFVIII product. Statistical summary available in *Online Supplementary Table S1*. (B) Lectin binding analysis of different production lots of full-length rFVIII from CHO or BHK cells. Data are representative of at least 3 independent experiments. BHK: baby hamster kidney cells; CHO: Chinese hamster ovary cells; rFVIII: recombinant factor VIII; BSA: bovine serum albumin; BDD: B-domain deleted.

in BHK cells, suggesting that differences in glycan profiles may be protein-specific.⁴⁰

Given the conflicting evidence relating to the role of mannosylation on FVIII immunity, and the high abundance of endogenous high-mannose glycans on other plasma proteins that do not exhibit similar immunogenic responses, we assessed sialic acid as a regulator of FVIII immunogenicity.^{15,16,41} N-glycans on BHK-rFVIII exhibited greater sialylation, which considering our clearance data, is in contrast to previously reported influences of sialic acid on FVIII and VWF half-life.⁴² The inhibition of com-

plex glycan formation has been shown to increase the specific activity of FVIII, suggesting that the enhanced negative charge due to increased sialic acid may alter the affinities between BHK-rFVIII and its interacting coagulant partners.⁴⁵ While the influence of the FVIII procoagulant activity on immunogenicity remains a subject of debate, the increased negative charge associated with enhanced sialylation may modulate clearance receptor binding.^{44,45}

Sialic acid can signal through inhibitory receptors such as Siglec-5, or the activating homolog Siglec-14, which have nearly identical binding motifs.^{14,46} In our study, we

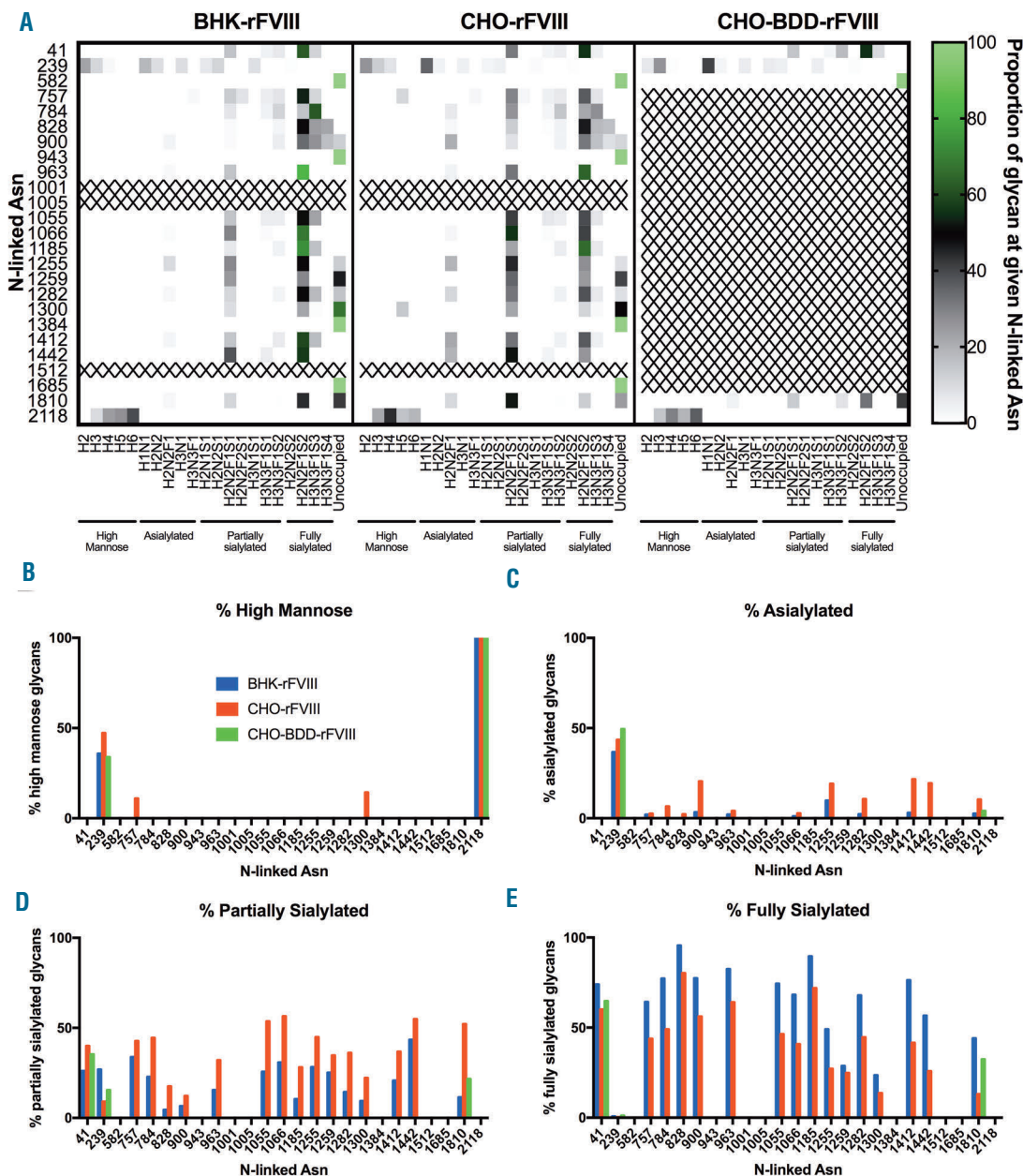


Figure 5. Comparative N-linked glycosylation of different rFVIII products by LC-MS/MS. (A) Heat plot representing the proportion of each of the detected glycans at each N-linked consensus sequence across different rFVIII products. Sites portrayed with an X were not detected in the analyses. Glycan structures are denoted as additions to the core (Man3GlcNAc2-Asn) where H=hexose, N=GlcNAc, F=fucose, S=sialic acid. (B) High-mannose, (C) asialylated, (D) partially sialylated, and (E) fully sialylated N-linked glycans presented as a proportion of the total occupied glycans. Amino acid numbering for N-linked Asn glycosylation sites does not include the 19 amino acid signal peptide. Each rFVIII product was analyzed at least twice, including an initial assessment of sample quality. BHK: baby hamster kidney cells; CHO: Chinese hamster ovary cells; rFVIII: recombinant factor VIII; BDD: B-domain deleted.

observed no difference in the binding of BHK- or CHO-rFVIII to naïve splenocytes or DCs. Moreover, the removal of α 2-3, and α 2-6 sialic acids did not significantly influence cellular binding or modulation of surface co-stimulatory molecule responses *in vitro*, and did not influence the immunogenicity of BHK-rFVIII in mice. In fact, the FVIII immune response was attenuated when exposed to desialylated BHK-rFVIII compared to exposure to unmodified rFVIII, potentially due to desialylated BHK-rFVIII behaving as a unique antigen.⁴⁷ Although the antigenicity of desialylated rFVIII was not altered as determined by

ELISA, the decrease in FVIII:C activity suggests an alteration of global tertiary structure that may influence FVIII immunogenicity.

Interactions between FVIII and its plasma binding partners may regulate its association with endocytic cells in the liver and spleen. We observed increased binding to BHK-rFVIII by non-neutralizing IgM in the plasma of naïve HA and HA-R593C mice as well as by non-neutralizing IgM and IgG from healthy human subjects. The presence of mouse and human VWF in these experiments suggests that these immune complexes can form in the cir-

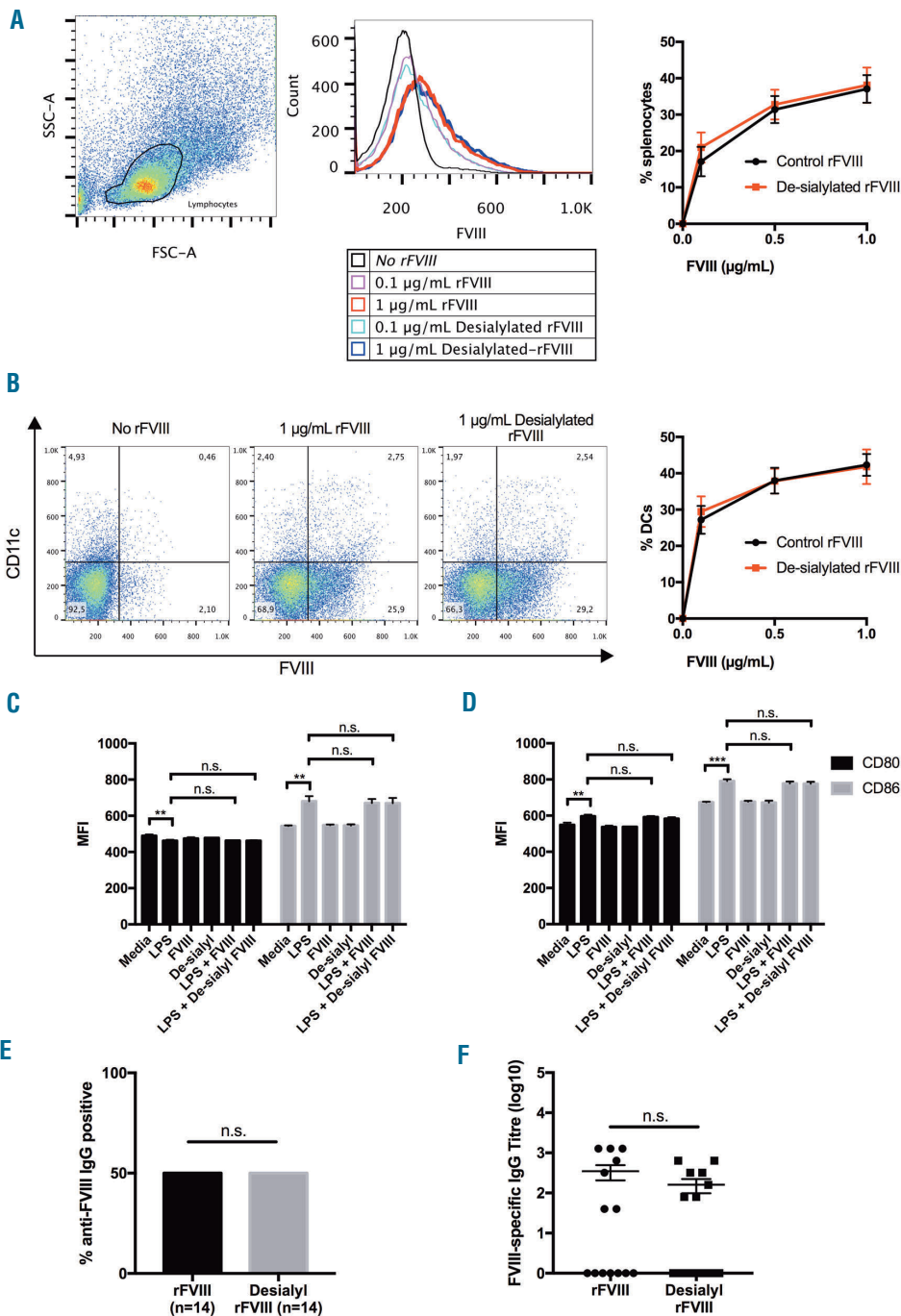


Figure 6. Sialic acid does not alter FVIII binding to, and maturation of splenocytes and dendritic cells *in vitro*, and does not modulate the FVIII immune response *in vivo*. Desialylated and control rFVIII were incubated with FVIII naïve splenocytes for 1 hr at 4 °C and assessed for binding by flow cytometry. Cells were gated as total (A) splenocytes or (B) CD11c⁺ dendritic cells (DCs). rFVIII association with DCs was quantified as the proportion of FVIII+CD11c⁺ out of all CD11c⁺ cells. Flow cytometry characterization of the surface co-stimulatory molecules, CD80 and CD86, were performed on (C) splenocytes and (D) CD11c⁺ DCs incubated for 24 hr under the indicated conditions (1 μ g/mL LPS or rFVIII; n=4). HA-R593C mice were administered 1 μ g of rFVIII and its desialylated glycoform subcutaneously biweekly for 2 weeks. Plasma samples 28 days after the initial infusion were assessed for (E) the incidence of FVIII-specific IgG and (F) the titre of the anti-FVIII IgG response. Line and error bars represent mean and SEM respectively. Data representative of at least 3 independent deglycosylation reactions. **P<0.01; ***P<0.001. MFI: mean fluorescence intensity; rFVIII: recombinant factor VIII; Ig: immunoglobulin; n.s. not significant.

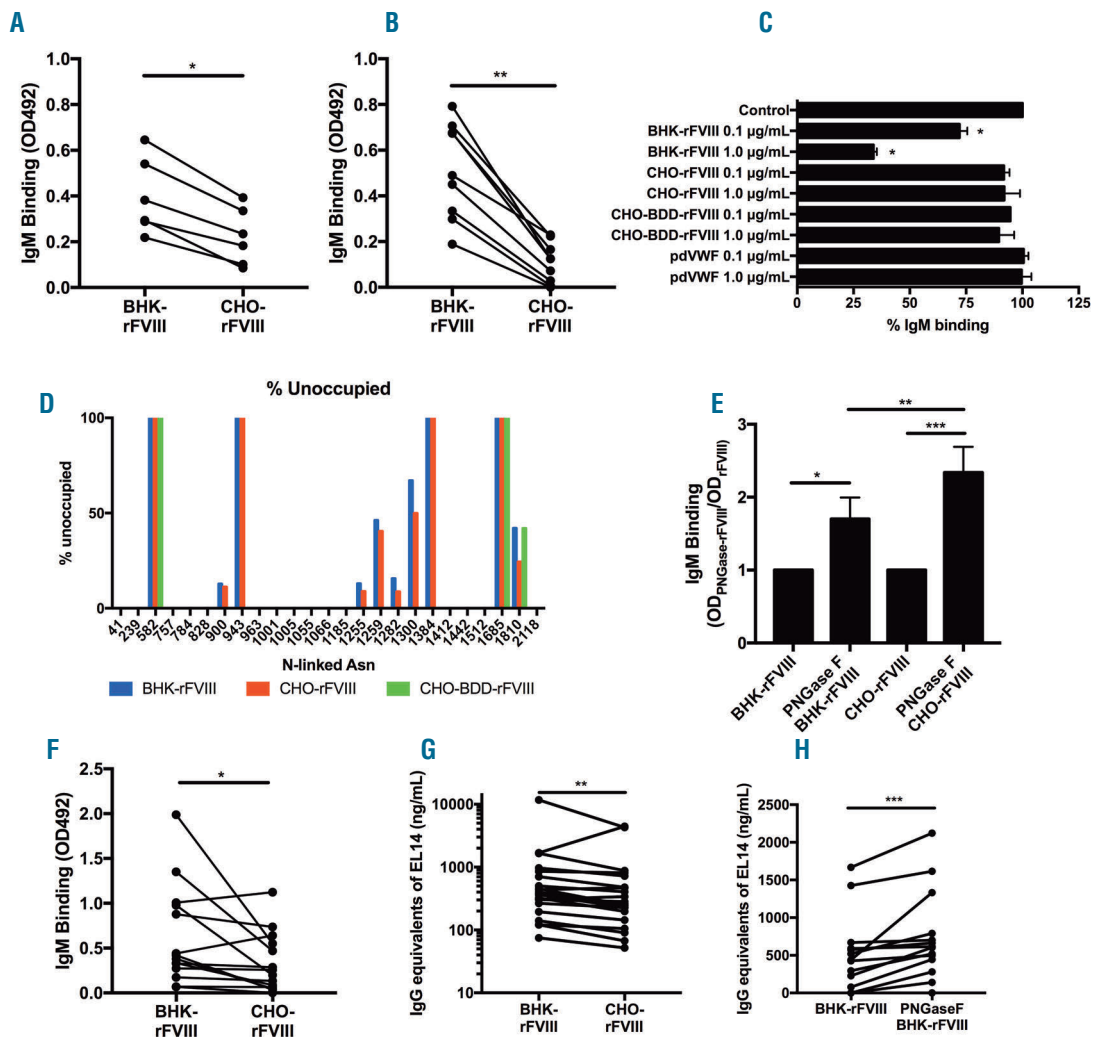


Figure 7. FVIII-specific IgM and IgG in naive HA mice and healthy humans bind preferentially to BHK-rFVIII. FVIII-specific IgM was detected by indirect ELISA using plasma from naive (A) HA-R593C mice and (B) HA mice. (C) Competition assay in which the specificity of these IgM antibodies for BHK-rFVIII was assessed by a 30 min preincubation with either other rFVIII concentrates, or human pdVWF (n=3). (D) Glycan occupancy at each N-linked Asn consensus sequence derived from LC-MS/MS analysis. (E) Murine IgM binding to deglycosylated rFVIII products (n=7). (F) FVIII-binding of IgM isolated from healthy humans. (G) Binding of IgG from healthy human plasma to different rFVIII products. (H) Binding of IgG from healthy human plasma to de-N-glycosylated BHK-rFVIII. Line and error bars represent mean and SEM respectively. The Wilcoxon test was used for paired statistical analysis. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. BHK: baby hamster kidney cells; CHO: Chinese hamster ovary cells; rFVIII: recombinant factor VIII; BDD: B-domain deleted; Ig: immunoglobulin; PNGaseF: Peptide: N-Glycosidase F.

ulation in the presence of the protective effect of VWF.⁴⁸ N-linked glycans may result in steric hindrance to prevent Ig binding. Indeed, removal of N-linked glycans from BHK- and CHO-rFVIII increased IgM binding to a greater extent in the latter, suggesting that glycans on CHO-rFVIII better mask underlying epitopes.

These rFVIII immune complexes may provide an explanation for the enhanced immunogenicity documented with BHK-rFVIII through fragment crystallizable (Fc)-mediated uptake by DCs.⁴⁹ The binding of IgM to antigens further facilitates the binding of mannose-binding lectin, and both can trigger the deposition of complement that greatly increases the binding potential of FVIII to endocytic receptors.⁵⁰ These data therefore support the observation of increased clearance and immunogenicity of BHK-rFVIII. The presence of FVIII-specific IgM in rFVIII naive HA mice, and both IgM and IgG in healthy individuals, suggests an innately immunogenic property of FVIII.

Previous studies have reported anti-FVIII antibodies in up to 19% of healthy subjects.^{26,27,51} These autoantibodies have been mapped to several regions in the FVIII heavy chain, and a single region in the A3 domain of the FVIII light chain.⁵¹ Complete glycosylation of the partially occupied sites described herein may inhibit these initial immune complexes from forming. Although these anti-rFVIII Igs likely possess low binding affinities, the avidity of IgM binding, in addition to IgG binding, may compensate and contribute to cellular uptake of FVIII immune complexes leading to either clearance or antigen presentation.^{52,53}

In this study, our data suggest that N-linked glycans shield underlying FVIII epitopes. We propose that the increased immunogenicity of BHK-rFVIII shown in two murine models of hemophilia A and four separate epidemiological studies is, in part, related to incomplete N-linked glycosylation that exposes immunogenic epitopes

to FVIII-specific IgM and IgG, that may, in turn, facilitate the formation of immune complexes in the circulation. Collectively, our studies provide an additional biological complement to evidence presented in recent epidemiological investigations showing that 2nd generation BHK-rFVIII is more immunogenic than 3rd generation CHO-rFVIII.

Funding

This work was supported by an Operating and Foundation Grant from the Canadian Institutes of Health Research (MOP-10912, FDN-154285), GlycoNet, and from the Canadian Hemophilia Society. JDL is supported in part by an Ontario Graduate Scholarship and the Franklin Bracken Fellowship. and DL is the recipient of a Canada Research Chair in Molecular Hemostasis.

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