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ORIGINAL ARTICLE

Quantitative HLA-class-II/factor VIII (FVIII) peptidomic variation in dendritic cells correlates with the immunogenic potential of therapeutic FVIII proteins in hemophilia A

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Abstract

Background: Plasma-derived (pd) or recombinant (r) therapeutic factor VIII proteins (FVIIIs) are infused to arrest/prevent bleeding in patients with hemophilia A (PWHA). However, FVIIIs are neutralized if anti-FVIII-antibodies (inhibitors) develop. Accumulating evidence suggests that pdFVIIIs with von Willebrand factor (VWF) are less immunogenic than rFVIIIs and that distinct rFVIIIs are differentially immunogenic. Since inhibitor development is T-helper-cell-dependent, human leukocyte antigen (HLA)-class-II (HLAcII) molecules constitute an important early determinant.

Objectives: Use dendritic cell (DC)-protein processing/presentation assays with mass-spectrometric and peptide-proteomic analyses to quantify the DP-bound, DQ-bound,

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and DR-bound FVIII-derived peptides in individual HLAcII repertoires and compare the immunogenic potential of six distinct FVIIIs based on their measured peptide counts.

Patients/Methods: Monocyte-derived DCs from normal donors and/or PWHA were cultured with either: Mix-rFVIII, a VWF-free equimolar mixture of a full-length (FL)-rFVIII [Advate® (Takeda)] and four distinct B-domain-deleted (BDD)-rFVIIIs [Xyntha® (Pfizer), NovoEight® (Novo-Nordisk), Nuwiq® (Octapharma), and Afstyla® (CSL Behring GmbH)]; a pdFVIII + pdVWF [Beriate® (CSL Behring GmbH)]; Advate ± pdVWF; Afstyla ± pdVWF; and Xyntha + pdVWF.

Results: We showed that (i) Beriate had a significantly lower immunogenic potential than Advate ± pdVWF, Afstyla − pdVWF, and Mix-rFVIII; (ii) distinct FVIIIs differed significantly in their immunogenic potential in that, in addition to (i), Afstyla + pdVWF had a significantly lower immunogenic potential than Beriate, while the immunogenic potential of Beriate was not significantly different from that of Xyntha + pdVWF; and (iii) rFVIIIs with pdVWF had significantly lower immunogenic potentials than the same rFVIIIs without pdVWF.

Conclusions: Our results provide HLAcII peptidomic level explanations for several important clinical observations/issues including the differential immunogenicity of distinct FVIIIs and the role of HLAcII genetics in inhibitor development.

KEYWORDS
blood coagulation factor inhibitors, factor VIII, hemophilia A, histocompatibility antigens class II, peptide, quantitative peptidomics, sequence analysis

1 | INTRODUCTION

Hemophilia A (HA) is the X-linked bleeding disorder resulting from FVIII gene (*F8*) mutations and deficient FVIII activity (see Table S1 for abbreviations). Infusions of pdFVIIIs or rFVIIIs are the standard of care for arresting and preventing bleeding in PWHA, but ~25% will develop neutralizing anti-FVIII-antibodies (inhibitors) that impair or eliminate their efficacy.[1] Patients with hemophilia A and inhibitors (PWHA/Inh+) have greater mortality/morbidity rates and require management with extremely expensive yet less safe/effective therapies. The pathogenesis of inhibitors is complex as it involves the multifaceted immune system and the immunologically-relevant characteristics of different FVIIIs and treatment strategies.[2] Moreover, in addition to these environmental variables, inhibitor risk is influenced by genetic variables[3] including the highly heterogeneous set of *F8* mutations[4]; functionally distinct single-nucleotide variations in immune response genes[5]; and haplotypes of nonsynonymous (ns)-single-nucleotide variations in genes that encode the (i) class-II (cII) HLA system, that is, *DPA1/DPB1*, *DQA1/DQB1*, and *DRA/DRB1/DRB3/DRB4/DRB5*,[6-9] and (ii) all or part of FVIII (i.e., *F8*, $F8_{I22I}$, and $F8_{B}$).[10-12] The immunogenicity risk of pdFVIIIs versus rFVIIIs has been studied/debated for years.[13] However, the randomized clinical trial SIPPET found that pdFVIIIs containing VWF, that is, pdVWF, are significantly less likely to elicit inhibitors—in severely affected previously untreated patients (PUPs)—than are rFVIIIs, which do not contain VWF.[14] These studies have been from a top-down perspective in which aggregate data over individuals are used to make inferences on molecular-level and cellular-level processes related to the immunogenicity of FVIIIs. Herein we present a complementary bottom-up approach based on an immune cell assay that identifies and quantifies the molecular determinants that initiate inhibitor development.[15-17]

The immune response to FVIIIs is initiated by antigen-presenting cells, primarily DCs,[18,19] via the following molecular/cellular processes.[15-19] After uptake, DCs proteolytically process FVIIIs into peptides. Some of the FVIII-derived peptides—often only a small

Essentials

- Antibody "inhibitors" of therapeutic FVIII proteins impair treatment of hemophilia A.
- HLA-class-II molecules are crucial early determinants in the pathogenesis of inhibitors.
- We used dendritic cells to identify and quantify the DP-bound, DQ-bound, and DR-bound FVIII-derived peptides in individuals.
- The immunogenic potential of FVIIIs is influenced by the variation in their HLAcII peptidomes.

subset of which will be "foreign" as the mutant *F8* in most patients expresses the amino acid-sequence of a full-length FVIII protein (FL-FVIII) in one or two aberrant polypeptides[11,20]—are then loaded into the binding grooves of one or more of the 3 to 12 distinct allotypes of DP, DQ, and DR isomers comprising individual HLAcII repertoires in unrelated subjects.[20-24] These HLAcII/peptide complexes are next translocated onto DC surfaces and presented to naïve CD4 T-cells. Because most HA-causing *F8* mutations express the amino acid-sequence of a FL-FVIII, that is, the endogenous material necessary for central tolerance induction, only a small fraction of the naïve T-cells in individual repertoires are likely to be FVIII-specific with an epitope comprising a "foreign" FVIII-derived-peptide bound to a "self" HLAcII molecule.[11,19-24] When engaging HLAcII/FVIII-derived-foreign-peptide complexes, these "primed" FVIII-specific CD4 T-cells undergo complete activation and differentiation into T-helper (T_H)-cells only upon receiving a "danger" signal from DCs also expressing CD80/86 costimulatory molecules.[2,19,25-27] Via these processes, DCs yield the effector T-cells that "help" activate FVIII-specific B-cells, which then proliferate and differentiate into anti-FVIII-antibody-secreting plasma cells.[2,19]

To begin characterizing the HLAcII-bound/FVIII-derived-peptidome and improve understanding of T-cell epitope generation/presentation, van Haren et al. pioneered the use of DC-protein processing/presentation assays (PPPAs) followed by mass-spectrometry and peptide-proteomic analyses,[28-31] together referred to as "major histocompatibility complex (MHC)-associated peptide-proteomics" (MAPPs) in our companion manuscript.[24] We now report results from our investigation of these processes—the FVIII Epitope Determination (FED) Study—that analyzed a pdFVIII and five distinct rFVIIIs in three independently performed DC-PPPAs.[21-24,32,33] The data generated from DC-PPPAs are the HLAcII-bound/FVIII-derived peptides identified, the number of which we predict is directly proportional to the immunogenic potential (IP) of a specific FVIII in a given PWHA.[22-24,32,33] Using a generalized-linear-mixed model to analyze the data organized into multiway contingency tables, we can make inferences regarding the relative importance to IP of the distinct FVIIIs; DP, DQ, and DR isomers; FVIII domains; and HLAcII genetics (alleles, genotypes, and haplotypes).

2 | MATERIALS AND METHODS

2.1 | Study design and therapeutic FVIII proteins

The pdFVIII studied originated as Beriate® (CSL Behring GmbH), which contains pdVWF (Figure S1) at concentrations varying lot to lot.[34] As described previously,[24,32] pdVWF in the combined reconstituted Beriate was first removed and the VWF-free pdFVIII (Figure 1) was DC-PPPA-tested as pdFVIII + pdVWF after add-back of pdVWF at 13.4-molar-excess using a FVIII-free pdVWF concentrate (see Formulations of FVIIIs).[22-24,32,33] The five rFVIIIs studied (Figure 1) originated as Advate® (Takeda), a full-length (FL) product termed FL-rFVIII,[35] and Xyntha® (Pfizer), NovoEight® (Novo-Nordisk), Nuwiq® (Octapharma), and Afstyla® (CSL Behring GmbH), engineered B-domain (BD)-deleted (BDD) products termed BDD-rFVIII$_1$, BDD-rFVIII$_2$, BDD-rFVIII$_3$, and BDD-rFVIII$_4$.[36-39] Figures S2 to S7 show the primary structures of these FVIIIs. See Formulations of FVIIIs for details on their preparations (± pdVWF) used in the DC-uptake assays (UAs) and the three independent DC-PPPAs performed. Chronologically these were DC-PPPA-Mix, DC-PPPA-S1, and DC-PPPA-S2, where "S" denotes use of a single FVIII per treatment. In DC-PPPA-Mix, we focused on optimizing the parameters of the experimental design, whereas in DC-PPPA-S1, we wanted to compare pdFVIII + pdVWF against rFVIIIs ± pdVWF, and in DC-PPPA-S2, we wanted to compare select FVIIIs + pdVWF in PWHA with and without inhibitors (see DC-PPPAs and MAPPs for more details on experimental design).

2.2 | Formulations of FVIIIs

After pdVWF removal from Beriate, using VIIISelect™ affinity purification resin (GE Healthcare) in a method detailed previously,[24,32] a mean specific activity of 5000 IU/mg was measured for the VWF-free pdFVIII with the Chromogenix Coamatic FVIII® activity assay (Diapharma). On the basis of mean specific activities of 7000 IU/mg for FL-rFVIII; 8100 to 10700 IU/mg for BDD-rFVIII$_1$, BDD-rFVIII$_2$, and BDD-rFVIII$_3$; and 12000 IU/mg for BDD-rFVIII$_4$, suitable amounts of each of these lyophilized rFVIIIs were reconstituted (per manufacturer's label) and concentrated using Amicon® stirred cells with Biomax® PES-membranes (Merck Millipore) to attain concentrations of 0.8 to 1.2 mg/mL. The integrity of all FVIIIs was confirmed by Chromogenix Coamatic FVIII activity assay (not shown) and SDS-PAGE (Figure S8) before DC-UA/DC-PPPA testing. Molecular weights of 170 and 280 kDa, respectively, were used to calculate molarities of the four BDD-rFVIIIs and two FL-FVIIIs (FL-rFVIII and pdFVIII).

CSL Behring provided an intermediate precipitate of the plasma fractionation process (Figure S9) that was the pdVWF source to make VWF-containing preparations of the pdFVIII, FL-rFVIII, and BDD-rFVIII$_4$ used in DC-PPPA-S1; and pdFVIII, FL-rFVIII, BDD-rFVIII$_1$, and BDD-rFVIII$_4$ used in DC-PPPA-S2. As previously described,[24,32] the pdFVIII was removed from the pdVWF via size-exclusion chromatography.[40] As shown in Figure S10 and explained in the Figure S9 legend, the first 16 size-exclusion fractions (1.A.2–2.A.2) assayed for VWF antigen (VWF:Ag) and FVIII:Ag were pooled because 1) together they contained >70% of the pdVWF in the original pd-concentrate, and 2) individually most (1.A.2–1.C.3) had no detectable pdFVIII and the last four (1.C.4–2.A.2) had only nominal to minimal pdFVIII. The VWF molarity in this concentrate—which contained negligible pdFVIII (<0.004% by mass)—was measured by ELISA (Agilent Dako) using a polyclonal rabbit anti-(human-VWF) capture antibody (A0082); horseradish peroxidase-conjugated detection antibody (P0226); and molecular weight of ~250 kDa for VWF monomers.

Using a subtly modified,[32] previously reported DC-UA[41] to test the FVIIIs—after preincubation with increasing pdVWF—we found the minimum molar excess of pdVWF to FVIII (i.e., 13.4:1) that maximally suppressed FVIII internalization (Figure S11). For relevant details of

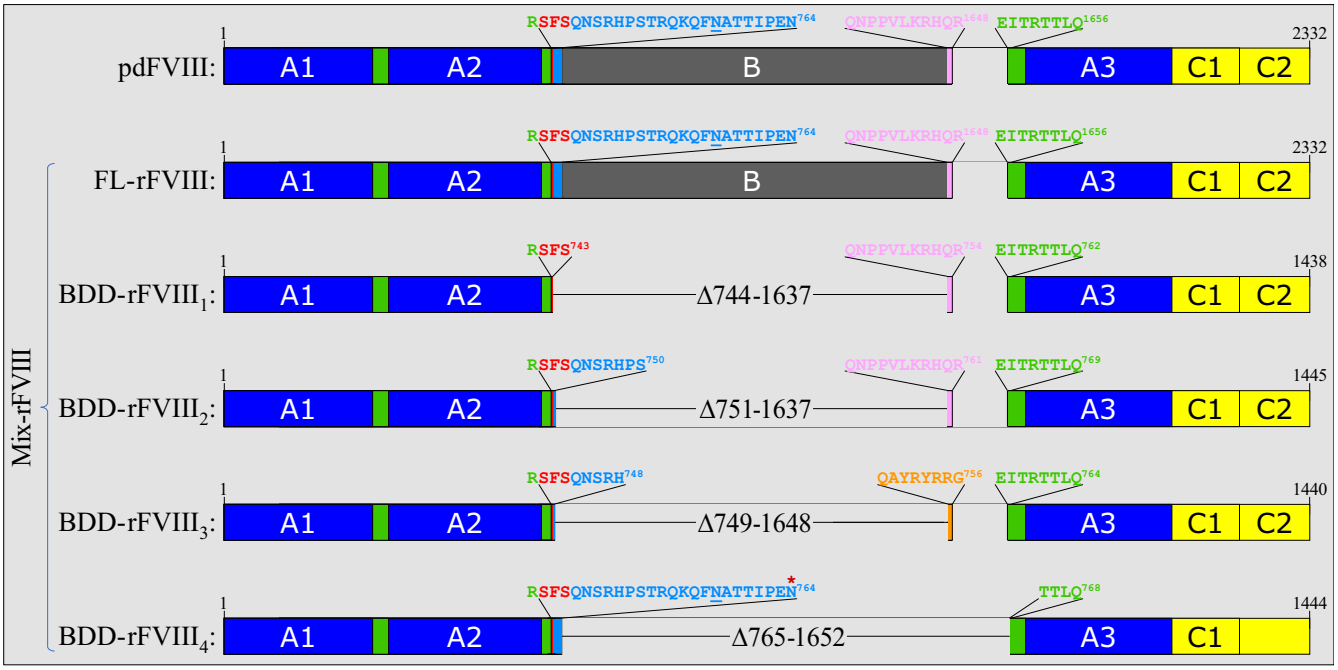


FIGURE 1 Schematic illustration of the distinct FVIIIs studied in DC-PPPAs. One pdFVIII and five rFVIIIs were studied to identify the FVIII-derived-peptides HLAcII-presented to T-cells. The rFVIIIs—whose amino acid sequences and other relevant characteristics are described in detail in the Supplemental Illustrations document (see Figures S2-S7)—included 1) Advate®, designated FL-rFVIII herein; 2) Xyntha®, designated BDD-rFVIII$_1$ herein; 3) NovoEight®, designated BDD-rFVIII$_2$ herein; 4) Nuwiq®, designated BDD-rFVIII$_3$ herein; and 5) Afstyla®, designated BDD-rFVIII$_4$ herein. As shown in Table 1, the FVIIIs used in: DC-PPPA-S1 were pdFVIII + pdVWF (#1), FL-rFVIII − pdVWF (#2), FL-rFVIII + pdVWF (#3), BDD-rFVIII$_4$ − pdVWF (#4), and BDD-rFVIII$_4$ + pdVWF (#5); DC-PPPA-S2 were pdFVIII + pdVWF (#1), FL-rFVIII + pdVWF (#3), BDD-rFVIII$_4$ + pdVWF (#5), and BDD-rFVIII$_1$ + pdVWF (#6); and DC-PPPA-Mix was Mix-rFVIII (#7), a VWF-free equal molar mixture of five rFVIIIs (i.e., FL-rFVIII, BDD-rFVIII$_1$, BDD-rFVIII$_2$, BDD-rFVIII$_3$, and BDD-rFVIII$_4$). While FL-rFVIII and BDD-rFVIII$_4$ were both used without and with pdVWF, pdFVIII and BDD-rFVIII$_1$ were used only with pdVWF, and Mix-rFVIII was used only without pdVWF. BDD-rFVIII$_4$, B-domain-deleted recombinant factor VIII$_4$; DC-PPPAs, DC-protein processing/presentation assays; FL-rFVIII, full-length recombinant factor VIII; FVIII, factor VIII; pdFVIII, plasma-derived factor VIII; pdVWF, plasma-derived von Willebrand factor

the modifications see the section DC-UAs as well as our companion manuscript[24] and the legend for Figure S11. We evaluated each FVIII by DC-PPPA at 146 nmol/L—whether tested alone or after preincubation with 13.4-fold molar excess pdVWF (i.e., 1956 nmol/L)—based on the 1) lower limits of MAPPs—established by ProImmune (Oxford, GBR)—to detect peptides in the ProPresent™ Antigen Presentation Assay, named DC-PPPA herein (see DC-PPPAs and MAPPs); and 2) amount of BDD-rFVIII$_1$ and BDD-rFVIII$_3$ available to us. Prior to analysis by DC-PPPA, 1956 nmol/L of pdVWF was combined with 146 nmol/L of either pdFVIII, FL-rFVIII, or BDD-rFVIII$_4$ (DC-PPPA-S1); or pdFVIII, FL-rFVIII, BDD-rFVIII$_1$, BDD-rFVIII$_4$ (DC-PPPA-S2). In DC-PPPA-S1, FL-rFVIII and BDD-rFVIII$_4$ were also analyzed at 146 nmol/L without pdVWF. Due to limiting numbers of peripheral blood mononuclear cells (PBMCs) from the normal donors (NDs) and PWHA used in DC-PPPA-S2, the FVIIIs were only analyzed with pdVWF.

2.3 | Cells

PBMCs were isolated from three NDs—Red Cross (Melbourne, Australia)—for use in DC-UAs. The PBMCs were then isolated from 28 NDs—ProImmune—and 6 PWHA, 4 without and 2 with inhibitors, Inh− and Inh+, respectively—University of North Carolina Chapel Hill (UNC)—for use in three independent DC-PPPAs (see DC-PPPAs and MAPPs). Our companion manuscript details relevant characteristics of the six PWHA for clinical/pathologic correlation.[24] After obtaining Institutional Review Board approval (No. 14-0582) and informed consent at UNC and Addenbrooke's Hospital (Cambridge, GBR), PBMCs were isolated from whole blood or component blood samples collected from each subject by peripheral venous phlebotomy or cytopheresis, respectively, as described previously.[24] The PBMCs from the 24 NDs used in DC-PPPA-S1 and DC-PPPA-Mix were obtained by peripheral venous phlebotomy at Addenbrooke's; and 6 PWHA and 4 NDs used in DC-PPPA-S2 were obtained by cytopheresis at UNC and Addenbrooke's, respectively. Our companion manuscript details the collection, processing, shipping, and DNA extraction of/from PBMCs.[24]

2.4 | HLAcII typing

The PBMC-derived DNA—from all 34 DC-PPPA-tested subjects—was used for typing HLAcII loci including *DPA1*, *DPB1*, *DQA1*, *DQB1*,

TABLE 1 FVIII-derived IPSs as immunologically relevant quantitative traits of the HLAcII/FVIII-peptidome

	Individual peptide sequences, IPSs (peptide counts)						
FVIII #:	1	2	3	4	5	6	7
Study ID (DC-PPPA-S1)							
D1030	2	113	56	85	8	NT	NT
D1098	15	120	44	50	5		
D1099	0	47	5	80	1		
D1111	53	207	68	101	2		
D1112	11	237	81	117	31		
D1123	49	172	86	163	56		
D1127	13	148	57	63	10		
D1128	34	94	121	53	8		
D1130	17	96	52	56	8		
D1131	20	119	127	90	8		
D1179	9	265	205	173	61		
D1184	18	191	77	164	16		
Study ID (DC-PPPA-S2)							
D1348	14	NT	2	NT	1	0	NT
D1447	1		1		0	5	
D1555	0		5		0	4	
D1563	0		3		0	0	
F801	55		96		40	52	
F802	4		29		0	11	
F803	42		63		22	49	
F805	0		19		0	0	
F8inh02	26		63		8	12	
F8inh03	30		81		7	33	
Study ID (DC-PPPA-Mix)							
D556	NT	NT	NT	NT	NT	NT	89
D632							234
D635							144
D636							238
D647							381
D656							109
D671							301
D675							222
D678							148
D679							238
D680							198
D693							278

The FVIIIs studied in: DC-PPPA-S1 were pdFVIII + pdVWF (#1), FL-rFVIII (#2), FL-rFVIII + pdVWF (#3), BDD-rFVIII$_4$ (#4), and BDD-rFVIII$_4$ + pdVWF (#5); DC-PPPA-S2 were pdFVIII + pdVWF (#1), FL-rFVIII + pdVWF (#3), BDD-rFVIII$_4$ + pdVWF (#5), and BDD-rFVIII$_1$ + pdVWF (#6); and DC-PPPA-Mix was Mix-rFVIII (#7), an equimolar mixture of five distinct rFVIIIs that included FL-rFVIII, BDD-rFVIII$_1$, BDD-rFVIII$_2$, BDD-rFVIII$_3$, and BDD-rFVIII$_4$. Not tested (NT).

Abbreviations: BDD-rFVIII$_4$, B-domain-deleted recombinant factor VIII$_4$; DC-PPPA, DC-protein processing/presentation assay; FL-rFVIII, full-length recombinant factor VIII; FVIII, factor VIII; pdFVIII, plasma-derived factor VIII; pdVWF, plasma-derived von Willebrand factor

DRB1, and, when present, *DRB3/4/5*. As described previously,[21-24,33] we used a next-generation sequencing assay (One Lambda) to sequence exons 2 to 6 of *DPB1*, *DQB1*, *DRB1*, and *DRB3/4/5*, and exons 1 to 5 of *DPA1* and *DQA1*. We report—at high-resolution (four-digit) level—the alleles encoding each subject's HLAcII repertoire based on the exon-2 sequences. To define the distinct molecules (allotypes) comprising each subject's HLAcII-based peptide antigen presentation repertoire, we established all unique combinations of the different α-polypeptide and β-polypeptide chains encoded by, respectively, their *DPA1* and *DPB1* alleles; *DQA1* and *DQB1* alleles; *DRA* and *DRB1/3/4/5* alleles. Since the monogenic α-chain and β-chain encoding DP and DQ loci are both polymorphic, the number of distinct DP and DQ allotypes both range between 1 and 4. Although most people have two functional *DRB* loci (i.e., *DRB1* and *DRB3/4/5*), their number of distinct DR allotypes ranges between 1 and 4 as the non-polymorphic *DRA* locus encodes the monomorphic α-chains of DR molecules.

2.5 | DC-UAs

As described in Formulations of FVIIIs, prior to DC-PPPA/MAPPs testing of the FVIIIs, we assessed the effect of pdVWF (presence vs. absence, and concentration) on their uptake by DCs—derived from monocytes of three NDs—using DC-UAs, which (as detailed previously[24,32] and in Figure S11) differed slightly from that described by Delignat et al.[41] Briefly, PBMCs were isolated—from buffy packs (Australian Red Cross; MSD #: 16-05VIC-24)—by ficoll histopaque gradient centrifugation per manufacturer (GE Healthcare). Monocytes were isolated—after washes and resuspension of 2×10^8 PBMCs in 1600 μL of purification buffer and 400 μL CD14 microbeads—using a MACS-LS column (Miltenyi) per manufacturer's instructions; and grown at 37°C with 5% CO_2 in RPMI-1640 media—containing 10% fetal bovine serum (FBS), 50 U/mL penicillin and 50 μg/mL streptomycin, 2 mmol/L Glutamax—in a 10-cm petri dish (1×10^7 cells/15 mL) with 500 IU IL-4/10^6 cells and 1000 IU GM-CSF/10^6 cells to induce DC differentiation. On day 6, harvested DCs were pelleted at 300 x *g* for 10 min, washed with prewarmed XVIVO media (Lonza), and plated (96-well round bottom plate; 2.5×10^5 cells/well/100 μL). Plates for analyzing FVIII uptake were incubated at 37°C with 5% CO_2; and cell surface binding (negative controls) were placed on ice. The six FVIIIs, all at 88.9 nmol/L, were separately incubated at 37°C for 10 min with either 2222.0 nmol/L, 1111.0 nmol/L, 555.5 nmol/L, or 0.0 nmol/L of pdVWF. These pdVWF-containing preincubated FVIIIs were added to the DCs in the 96-well plates, which were 1) incubated at either 37°C or 4°C for 2 h; 2) centrifuged at 300 x *g* for 5 min; 3) washed with fluorescence-activated cell sorter buffer (phosphate-buffered saline and 2% FBS); and 4) resuspended in 100 μL of IntraPrep Reagent 1 (Beckman Coulter). After allowing fixation at 25°C for 15 min, DCs were spun down at 300 x *g* for 5 min; washed with 200 μL of fluorescence-activated cell sorter buffer; resuspended with 100 μL of IntraPrep Reagent 2 (Beckman Coulter); incubated at 25°C for 5 min; treated with 10 μg/mL of

an A2-domain-specific anti-FVIII-antibody (Thermo Fisher: MA1-27389) for 15 min; pelleted, washed with fluorescence-activated cell sorter buffer, and stained with 50 μL of a 1:100 diluted anti-mouse-immunoglobulin-G detecting antibody (Jackson) in the dark at 25°C for 15 min; pelleted, washed with fluorescence-activated cell sorter buffer and analyzed by flow cytometry as described in Figure S11, which shows data for internalization of FL-rFVIII, BDD-rFVIII$_1$, and BDD-rFVIII$_4$ by the DCs from the three NDs [mean ± standard error of the mean (SEM)]; data for BDD-rFVIII$_2$, BDD-rFVIII$_3$, and pdFVIII are not shown.

2.6 | DC-PPPAs and MAPPs

DC-protein processing/presentation assays (PPPAs) were performed—as detailed in our companion manuscript[24]—to identify HLAcII-bound/FVIII-derived-peptides presented by DCs after the uptake and processing of FVIIIs. These analyses were performed by ProImmune using the ProPresent Antigen Presentation Assay (for additional details, see Xue et al., Ventura et al., and Gouw et al.[42-44]). Using DCs obtained from three different cohorts (see Cells) in independent DC-PPPAs under distinct experimental conditions (i.e., S1, S2, and Mix), immature DCs were cultured with 146 nmol/L of FL-rFVIII or BDD-rFVIII$_4$ ± 1956 nmol/L pdVWF in DC-PPPA-S1; 146 nmol/L pdFVIII + 1956 nmol/L pdVWF in DC-PPPA-S1 and DC-PPPA-S2; 146 nmol/L of FL-rFVIII, BDD-rFVIII$_1$, or BDD-rFVIII$_4$ + 1956 nmol/L pdVWF in DC-PPPA-S2; or 146 nmol/L of Mix-rFVIII—an equimolar mixture with 29.2 nmol/L each of FL-rFVIII, BDD-rFVIII$_1$, BDD-rFVIII$_2$, BDD-rFVIII$_3$, and BDD-rFVIII$_4$—without pdVWF in DC-PPPA-Mix. After the DCs were matured, harvested, washed, and detergent-lysed, their HLAcII molecules were affinity purified as three separate isomer fractions with proprietary anti-DP, anti-DQ, and anti-DR monoclonal antibodies whose binding properties were established by ProImmune (www.ProImmune.com) to be equivalent to the mouse monoclonal antibodies used previously in the setting of DC-PPPAs with MAPPs analysis,[15,28-31,42-44] which include anti-DP (B7/21) (Leinco Technologies); anti-DQ (SPV-L3) (Novus Biologicals); and anti-DR (L243) (Abcam). Peptides eluted from the DP-peptide, DQ-peptide, and DR-peptide complexes were analyzed by high-resolution sequencing mass spectrometry (MS), that is, liquid chromatography (LC) tandem MS (LC-MS/MS). The set of FVIII-derived individual peptide sequences (IPSs) were identified by peptide-proteomics analysis using software to compare the resulting MS data against the Uniprot Swiss Prot Reference Human Proteome Database supplemented with the: non-native BDD-junction sequences in the four engineered FVIIIs (Figures S4-S7); and minor alleles of the 56 known *F8* ns-single-nucleotide variants/-single-nucleotide polymorphisms due to (i) the possible presence of one or more of these naturally occurring residues in the FL-FVIIIs comprising Beriate (Figure S2), and (ii) the presence of the E-allele of ns-single-nucleotide polymorphism 1241D>E in FL-rFVIII (Figure S3).[10,20,35]

As detailed previously,[24] quality control of the three DC-PPPA/MAPPs experiments performed involved confirming that the immature and mature DCs expressed DC-markers CD86, DC-SIGN, and DR isomers of human leukocyte antigens (HLA-DR) using immunocytochemistry and flow cytometry (data not shown); and identifying (in the separate DP-peptidomes, DQ-peptidomes, and DR-peptidomes analyzed) HLAcII-bound-IPSs derived from endogenous proteins known to reside in the endoplasmic reticulum, Golgi, and/or endolysosomal compartment. Specifically, for each of the three isomer groups of HLAcII molecules in a given experiment—for example, the DR-peptidome of a certain subject's DCs cultured with a specific FVIII—to pass quality control, within the collection of IPSs identified some had to have arisen from a minimum of three of the following six such endogenous proteins: invariant chain; lysosome associated membrane proteins-1/-3; transferrin receptor; FCER2/FCGR2; integrin α_M; and apolipoprotein B.[45] The likelihood of a LC-MS/MS-identified-peptide being a real identity is described by its expect value and the false discovery rate.[42,46] Our companion manuscript[24] describes how the 1) scoring algorithms and statistical significance determinations were used; 2) residues in FVIII-derived-IPSs were numbered if they originated from an engineered rFVIII in or downstream of its BDD-junction; and 3) FVIII-derived-IPSs were counted if they contained a (i) non-native BDD-junction-sequence or (ii) minor allele(s) at a variable residue(s). To replicate the DC-PPPAs/MAPPs analyses conducted herein, a request may be submitted to ProImmune to perform ProPresent assays using the same FVIIIs and experimental conditions, and similar cellular samples from comparable NDs and PWHA.

2.7 | Log-linear model analysis

We performed a log-linear mixed effects model analysis of multiway contingency tables of the data in the R-package called "glmm" that accounted for potential sources of non-independence by modeling random effect variance components as appropriate (see Tables S2-S4 for more details).[47,48] With the regression coefficient estimates (betas) and their standard errors, we can construct risk ratios and their 95% confidence intervals (95% CIs).[47,48] For a given beta and its standard error denoted by beta_SE, we write:

$$\text{risk ratio} \pm 95\%\,\text{CI} = \exp(\text{beta}) \pm \exp(\text{beta} \pm 1.96 * \text{beta\_SE})$$

We analyzed three models to predict the natural logarithm of peptide counts, the dependent variable in all cases. For Model 1, the independent predictors are specific FVIII, HLAcII haplotype, HLAcII isomer, and FVIII domain. To account for potential non-independence due to donor, we modeled donor as a random effect variable. Model 1 was used to analyze all data from DC-PPPA-S1. For Model 2, the independent predictors are specific FVIII, DRB1 alleles, HA status, and FVIII domain. For this model, we used two random effect variables to account for potential non-independence due to donors and experiments. Model 2 was used to analyze only the DR-bound-peptides

presented in DC-PPPA-S1, DC-PPPA-S2, and DC-PPPA-Mix. For Model 3, the independent predictors are specific FVIII, inhibitor status, DRB1 genotypes, and FVIII domain. We again accounted for potential non-independence due to donors. Model 3 was used to analyze the DR-bound-peptide data for DC-PPPA-S2. Model 1 differs from Models 2 and 3—in which only DR-bound-peptides were analyzed—in that peptides from all three HLAcII isomers were analyzed (i.e., isomers head-to-head compared). Model 2 is the only one that analyzed peptides from all FVIIIs, which were studied across the three DC-PPPAs (i.e., therapeutics head-to-head compared). Model 3 differs from Models 1 and 2 in that it is the only one that analyzed peptides from PWHA (HA-/inhibitor-status head-to-head compared). This analysis strategy was necessary because when all of the data were analyzed together—in one model—there were too many cells with zero peptide counts, which caused failure of the estimation algorithm.

2.8 | Predicted affinities of the DP, DQ, and DR allotypes for the FVIII-derived peptides identified

As described in our companion manuscript,[24] we used netMHCIIpan-3.2 to predict the affinity of each FVIII-derived IPS—identified by MAPPs among the naturally processed peptides eluted from the DP, DQ, and DR isomers of DCs evaluated in DC-PPPA-S1, DC-PPPA-S2, and DC-PPPA-Mix—for the appropriate allotype(s) in the individual HLAcII-repertoires studied (Tables S5-S7).[49]

3 | RESULTS

3.1 | Quantitative variation in HLAcII/FVIII-derived peptidomes

We characterized the HLAcII/FVIII peptidomes—that is, the portion of a subject's HLAcII-bound-IPSs derived from a given FVIII and DC-PPPA—of 28 NDs and 6 PWHA by MAPPs analysis including peptides with 1) a non-native BDD-junction sequence (Figures 1 and S4-S7) or 2) a native sequence spanning the minor allele of a known ns-single-nucleotide polymorphism and/or ns-single-nucleotide variant (Figures S2 and S3). As shown in Table 1 and Tables S5 to S7, for the three independently performed DC-PPPAs (S1, S2, and Mix), we observed substantial quantitative variation between subjects and across FVIIIs both in the specific portions of FVIII that are HLAcII presented (coverage) and depth at which any given FVIII segment is HLAcII presented (density). In Table 1, we report the descriptive statistics of the FVIII-derived-IPSs identified. In Table S8, we report the descriptive statistics of the groups of overlapping peptides (GOPs) compiled. A single group of overlapping peptides (GOP) represents the set of overlapping IPSs from one FVIII segment. Figure 2A shows the domain structure of FL-FVIII in relation to the quantitative MAPPs data shown in Figures 2B to 2D, which, respectively, depict locations of the GOPs from DC-PPPA-Mix, DC-PPPA-S1, and all three DC-PPPAs combined. The black boxes in Figures 2B and 2C depict the location of the GOPs, broken down by HLAcII isomer (DP, DQ, and DR), from a representative ND for the appropriate sample, where the adjacent number represents the number of IPSs in each GOP.

3.2 | Log-linear analysis of aggregate peptidomic data

The most efficient approach to evaluate variables implicated in inhibitor risk was to analyze these data in aggregate presented in the form of multiway contingency tables (Tables S2-S4) using a log-linear model. Under this model, we can compute risk ratios for each independent predictor variable or variable factor (in the case of categorical variables with more than two levels). For categorical independent variables consisting of two levels, the risk ratio is interpreted in terms of the presence or absence of the variable such as the presence/absence of a given HLA allele (see later discussion). For categorical variables consisting of more than two levels, such as FVIII type, the risk ratios are computed against a reference baseline. To interpret the risk ratios, it should be recalled that the dependent variable in all models is the natural logarithm of HLAcII-bound/FVIII-derived-peptide counts. For a given FVIII level representing a specific FVIII, a risk ratio significantly <1 means that this FVIII contributes significantly less to the FVIII-derived-peptide count than the baseline FVIII, and vice versa, if the risk ratio is significantly >1. If the risk ratio is not significantly different from 1, then the given FVIII does not contribute any more or any less than the baseline.

3.3 | PdFVIII is HLAcII-presented significantly less frequently than FL-rFVIII ± pdVWF

The SIPPET study found that PUPs with severe HA administered pdFVIIIs with pdVWF developed inhibitors significantly less often than those given rFVIIIs. Because all rFVIIIs lack VWF, we directly compared FL-rFVIII to pdFVIII + pdVWF for their presentation by DCs on the DP, DQ, and DR isomers in the individual HLAcII repertoires of the 12 NDs tested in DC-PPPA-S1, which were comprised of by as few as 3 to as many as 12 distinct HLAcII allotypes (Figure 3A, "2"). For the risk ratios discussed under this section, the baseline is always pdFVIII + pdVWF, designated "1". The risk ratio for this first comparison is 3.7 with a 95% CI of 3.3 to 4.2. Hereon we quote the risk ratio and 95% CI as risk ratio (95% CI lower bound, 95% CI upper bound). Similarly, the risk ratio for FL-rFVIII + pdVWF, while less, is 2.2 (1.9, 2.5) (Figure 3A, "3"). The same patterns hold when we analyze the combined DR-bound/FVIII-derived-peptide counts from DC-PPPA-S1, DC-PPPA-S2, and DC-PPPA-Mix (Figure 3B, "2" and "3") with corresponding risk ratios of 2.6 (2.4, 2.9) and 1.6 (1.5, 1.8) for FL-rFVIII and FL-rFVIII + pdVWF. This pattern of pdFVIII + pdVWF yielding significantly lower HLAcII-presented/FVIII-derived-peptide counts is upheld even when we restrict the analysis to the DR-bound/

FVIII-derived-peptides from the PWHA in DC-PPPA-S2. Here we found a risk ratio of 1.3 (1.1, 1.6) for the FL-rFVIII + pdVWF to pdFVIII + pdVWF comparison (Figure 3C, "2").

3.4 | Engineered rFVIIIs are not equally HLAcII presented by DCs

We also compared pdFVIII + pdVWF to other rFVIIIs, namely, BDD-rFVIII$_1$ + pdVWF, BDD-rFVIII$_4$ ± pdVWF, and the equimolar mixture of rFVIIIs (Mix-rFVIII). Figure 3A ("4") shows that BDD-rFVIII$_4$ yielded a significantly higher HLAcII-presented-peptide count, relative to the pdFVIII + pdVWF baseline, with a risk ratio of 3.0 (2.7, 3.4). For this comparison, the pattern is upheld in Figure 3B ("4"), which shows a risk ratio of 2.2 (2.0, 2.5) for BDD-rFVIII$_4$ relative to baseline. Notably, as shown in Figures 3A ("5"), 3B ("5"), and 3C ("3"), when BDD-rFVIII$_4$ + pdVWF was compared to the pdFVIII + pdVWF baseline, it yielded significantly lower HLAcII-presented-peptide counts with risk ratios of 0.76 (0.65, 0.91), 0.63 (0.55, 0.73) and 0.56 (0.42, 0.74), respectively. Moreover, as can be seen in Figures 3B ("6") and 3C ("4"), BDD-rFVIII$_1$ + pdVWF does not differ significantly from pdFVIII + pdVWF with risk ratios of 1.17 (0.96, 1.41) and 0.98 (0.78, 1.23), respectively. As expected, Mix-rFVIII, which lacked pdVWF, yielded significantly higher HLAcII-peptide counts than pdFVIII + pdVWF (Figure 3B, "7").

The preceding comparisons suggested the question of how BDD-rFVIII$_4$ + pdVWF would compare with the other rFVIIIs, where BDD-rFVIII$_4$ + pdVWF was the baseline. We found that FL-rFVIII + pdVWF yielded significantly greater HLAcII-presented/FVIII-derived-peptide counts than BDD-rFVIII$_4$ + pdVWF [Figures 3A ("6") and 3C ("5")] with corresponding risk ratios of 2.86 (2.47, 3.32) and 2.33 (1.79, 3.02). Similarly, we found that BDD-rFVIII$_1$ + pdVWF also yielded significantly greater HLAcII-presented-peptide counts than BDD-rFVIII$_4$ + pdVWF (Figure 3C, "6") with a risk ratio of 1.76 (1.33, 2.32).

Figures 3A and 3B reveal that coadministering pdVWF with a rFVIII results in significantly less HLAcII-peptide-presentation. Using the model parameters and the fact that both BDD-rFVIII$_4$ and FL-rFVIII were compared to the same baseline of pdFVIII + pdVWF, we find the risk ratios for BDD-rFVIII$_4$ + pdVWF compared to BDD-rFVIII$_4$ − pdVWF and for FL-rFVIII + pdVWF compared to FL-rFVIII − pdVWF to be 0.26 (0.21, 0.31) and 0.59 (0.50, 0.70), respectively. Thus, pdVWF has a protective effect on both rFVIIIs, and its effect on BDD-rFVIII$_4$ is more pronounced than on FL-rFVIII.

3.5 | HLAcII alleles, genotypes, and haplotypes modulate IP especially those involving DRB1*15:01

Initial inspection of the data revealed that the DQB1*06:02/DRB1*15:01 haplotype had the highest HLAcII-presented/FVIII-derived-peptide counts overall, relative to the other HLAcII haplotypes, making it the choice for baseline haplotype "level". We compared it with three other haplotypes comprising >90% of the peptides, namely, DQB1*02:01/DRB1*03:01, DQB1*03:01/DRB1*11:01, and DQB1*03:02/DRB1*04:01. These haplotypes were all associated with significantly lower HLAcII/FVIII-peptide counts, relative to baseline, with risk ratios of 0.33 (0.31, 0.36), 0.15 (0.13, 0.17), and 0.55 (0.52, 0.59), respectively (Figure 4A).

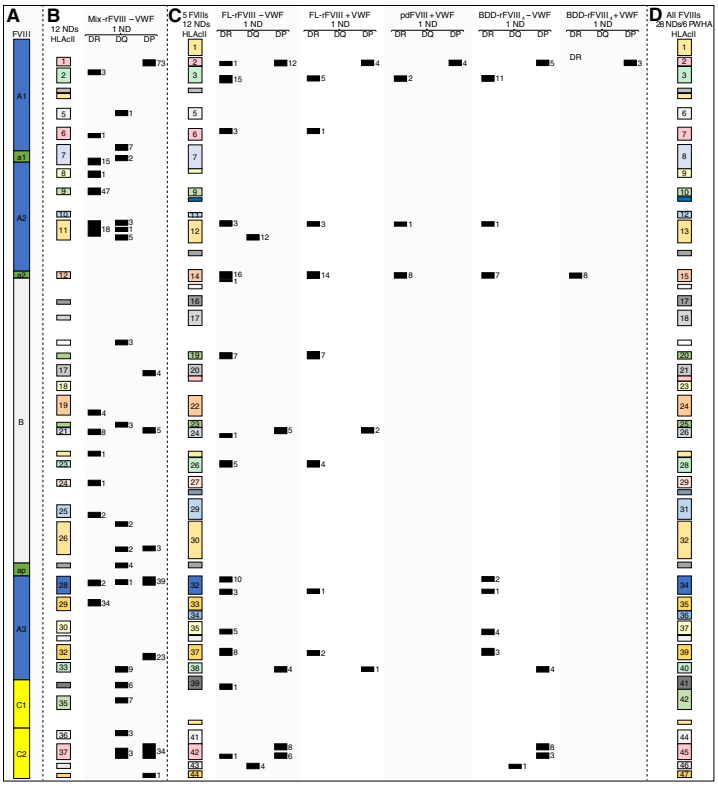
In Figure 4B, we report the risk ratios for four different DRB1 alleles—namely, DRB1*03:01, DRB1*04:01, DRB1*04:04, and DRB1*15:01—that accounted for ~90% of the data across all alleles.

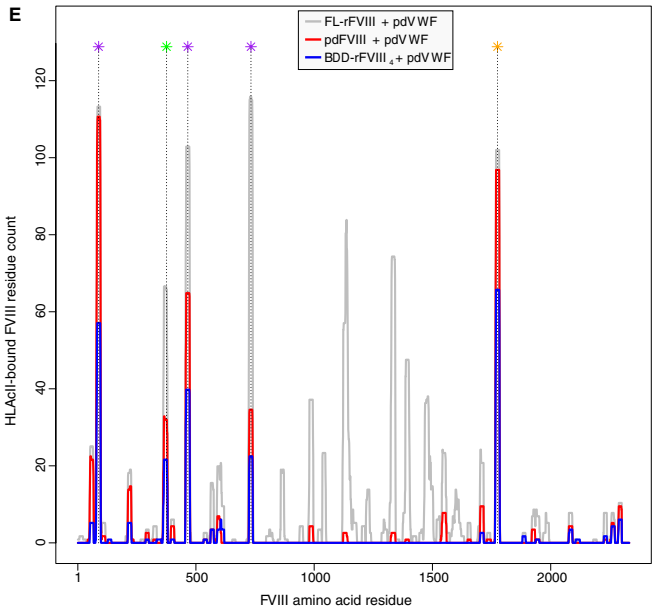
FIGURE 2 A, Domain structure of the 2332 amino acid-residue containing wild-type full-length FVIII protein (FL-FVIII). B, Total number and distribution of all groups-of-overlapping-peptides (GOPs) determined from the HLAcII-bound IPSs derived from Mix-rFVIII (− pdVWF) after incubation with DCs from either (i) any of the 12 NDs used in DC-PPPA-Mix (combined), with the GOPs shown as solid rectangles of assorted colors and different lengths based on the N-terminal-most and C-terminal-most residues of the FVIII-IPSs defining a given GOP; or (ii) a representative ND (ID#: D647) used in DC-PPPA-Mix (individual), with GOPs shown as solid black rectangles of different lengths based on the N-terminal and C-terminal residues of the FVIII-IPSs defining any given GOP. Whether considering the combined or individual GOPs data, the FVIII-IPSs underlying these GOPs were determined by LC-MS/MS and peptide-proteomics after elution from one or more of the distinct HLAcII allotypes (whether DP, DQ, and/or DR) comprising either the combined antigen-presentation repertoires of all 12 NDs or the individual antigen-presentation repertoire of D647. C, Total number and distribution of all GOPs determined from the HLAcII-bound IPSs derived from any of the five FVIIIs tested in DC-PPPA-S1 (pdFVIII + pdVWF, FL-rFVIII ± pdVWF, and BDD-rFVIII$_4$ ± pdVWF) after incubation with DCs from either (i) any of the 12 NDs used (combined), with the GOPs shown as solid rectangles of assorted colors and different lengths based on the N-terminal-most and C-terminal-most residues of the FVIII-IPSs defining a given GOP; or (ii) a representative ND (ID#: D1098) used, with the GOPs shown as solid black rectangles of different lengths based on the N-terminal-most and C-terminal-most residues of the FVIII-IPSs defining any given GOP. D, Total number and distribution of all GOPs (solid rectangles of assorted colors and different lengths based on the N-terminal-most and C-terminal-most residues of the FVIII-IPSs defining any given GOP) determined from the HLAcII-bound IPSs derived from any of the FED study's seven FVIIIs tested in either (i) DC-PPPA-S1, after incubation with DCs from any of the 12 NDs used (pdFVIII + pdVWF, FL-rFVIII ± pdVWF, or BDD-rFVIII$_4$ ± pdVWF); (ii) DC-PPPA-Mix, after incubation with DCs from any of the 12 NDs used (Mix-rFVIII − pdVWF); or (iii) DC-PPPA-S2, after incubation with DCs from any of the four NDs and 6 PWHA used (pdFVIII + pdVWF, FL-rFVIII + pdVWF, BDD-rFVIII$_1$ + pdVWF, and BDD-rFVIII$_4$ + pdVWF). E, Plot of the HLAcII-bound FVIII residue counts against the FVIII amino acid-residues derived from FL-rFVIII + pdVWF (gray line), pdFVIII + pdVWF (red line), and BDD-rFVIII$_4$ + pdVWF (blue line) for all HLAcII-bound fractions (i.e., DP, DQ, and DR) in DC-PPPA-S1 and the HLA-DR-bound fraction in DC-PPPA-S2. BDD-rFVIII$_4$, B-domain-deleted recombinant factor VIII$_4$; DC-PPPA, DC-protein processing/presentation assay; GOP, group of overlapping peptides; HLAcII, human leukocyte antigen class II; IPS, individual peptide sequence; FL-rFVIII, full-length factor VIII; FVIII, factor VIII; pdFVIII, plasma-derived factor VIII; pdVWF, plasma-derived von Willebrand factor

Respectively, we found risk ratios of 1.05 (0.96, 1.15), 1.50 (1.35, 1.66), 2.31 (2.13, 2.50), and 1.61 (1.50, 1.73). Thus, DRB1*03:01 is the only one of these four alleles that is not significantly associated with HLAcII-bound/FVIII-derived-peptide counts.

Figure 4C shows the effects of three DRB1 genotypes with the DRB1*15:01 allele relative to the baseline DRB1*04:01/DRB1*13:02 genotype. Two of these genotypes yielded significantly greater HLAcII/FVIII-derived-peptide counts (DRB1*07:01/DRB1*15:01 & DRB1*04:08/DRB1*15:01) whereas DRB1*04:07/DRB1*15:01 yielded significantly lower HLAcII/FVIII-derived-peptide counts.

Our results regarding DRB1*15:01 yielded another notable finding. To understand this finding, we need to emphasize that we had assumed that, all else being equal, DCs from NDs would not differ from DCs from PWHA in their biology underlying protein uptake, processing, and presentation. We made a similar assumption concerning DCs from PWHA/Inh− and PWHA/Inh+. However, as seen in Figure 5A, DCs from PWHA/Inh− and PWHA/





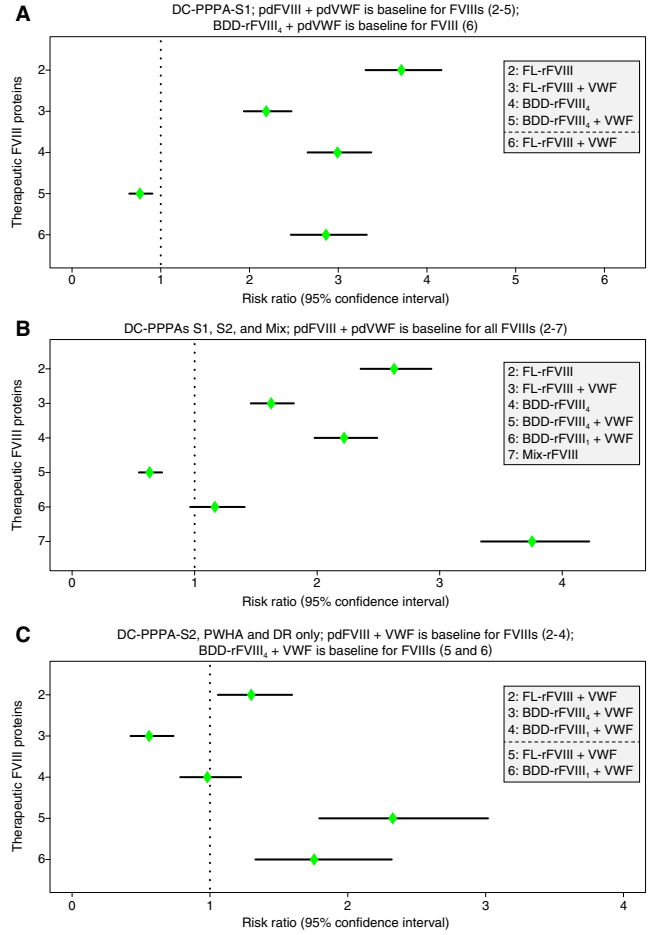


FIGURE 3 Effect of distinct FVIIIs on DC FVIII-peptide presentation. Risk ratios (green diamonds) and 95% confidence intervals (black lines) for the FVIIIs analyzed in Models 1-3 (see text). A, Model 1: pdFVIII + pdVWF was baseline for FL-rFVIII − pdVWF (2), FL-rFVIII + pdVWF (3), BDD-rFVIII$_4$ − pdVWF (4), and BDD-rFVIII$_4$ + pdVWF (5); BDD-rFVIII$_4$ + pdVWF was baseline for FL-rFVIII + pdVWF (6). B, Model 2: pdFVIII + pdVWF was baseline for FL-rFVIII − pdVWF (2), FL-rFVIII + pdVWF (3), BDD-rFVIII$_4$ − pdVWF (4), BDD-rFVIII$_4$ + pdVWF (5), BDD-rFVIII$_1$ + pdVWF (6), and Mix-rFVIII − pdVWF (7). C, Model 3: pdFVIII + pdVWF was baseline for FL-rFVIII + pdVWF (2), BDD-rFVIII$_4$ + pdVWF (3), and BDD-rFVIII$_1$ + pdVWF (4); BDD-rFVIII$_4$ + pdVWF was baseline for FL-rFVIII + pdVWF (5) and BDD-rFVIII$_1$ + pdVWF (6). FVIII, factor VIII protein; pd, plasma derived; VWF, von Willebrand factor; r, recombinant; FL-rFVIII, full-length rFVIII; BDD-rFVIII, B-domain-deleted-rFVIII; DC, dendritic cell; DC-PPPAs, DC-protein processing/presentation assays

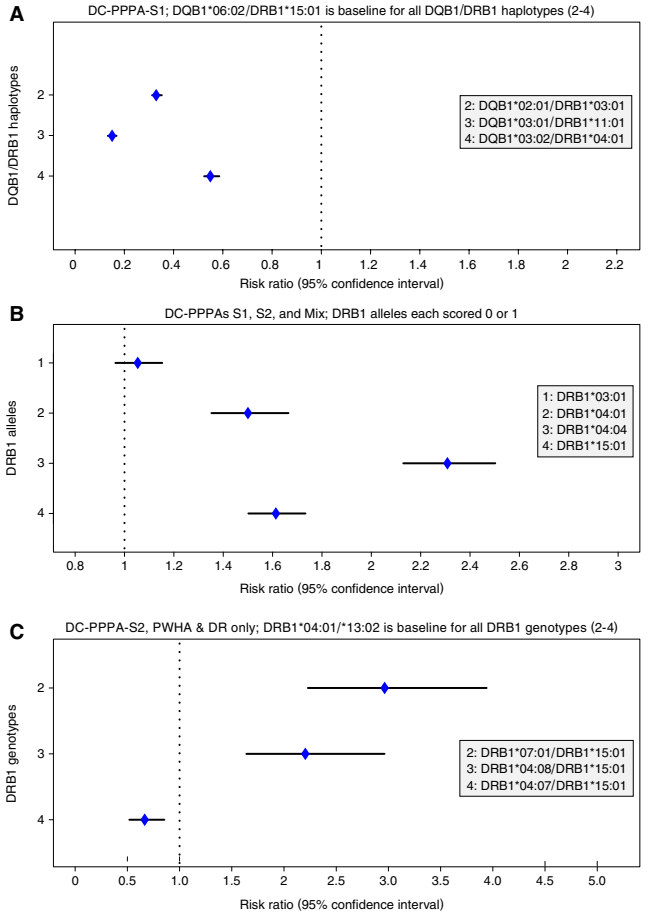


FIGURE 4 Effect of HLAcII haplotypes, genotypes, and alleles on DC FVIII-peptide presentation. Risk ratios (blue diamonds) and 95% confidence intervals (black lines) for the HLA parameters analyzed in Models 1-3 (see text). A, Model 1, DQB1/DRB1 haplotypes: DQB1*06:02/DRB1*15:01 was baseline for DQB1*02:01/DRB1*03:01 (2), DQB1*03:01/DRB1*11:01 (3), and DQB1*03:02/DRB1*04:01 (4); B, Model 2, DRB1 alleles: DRB1*03:01 (1), DRB1*04:01 (2), DRB1*04:04 (3), and DRB1*15:01 (4) are treated as dichotomous variables; and C, Model 3, DRB1 genotypes: DRB1*04:01/DRB1*13:02 was baseline for DRB1*07:01/DRB1*15:01 (2), DRB1*04:08/DRB1*15:01 (3), and DRB1*04:07/DRB1*15:01 (4)

Inh+ presented significantly more HLAcII-bound/FVIII-derived-peptides than DCs from NDs (i.e., the baseline) with respective risk ratios of 1.99 (1.12, 3.53) and 5.99 (3.49, 10.26). Furthermore, as Figure 5B reveals, PWHA/Inh− presented significantly fewer HLAcII/FVIII-derived-peptides than PWHA/Inh+ with a risk ratio of 0.33 (0.25, 0.46). At face value, these results would seem to reject our working hypotheses, but on closer inspection we found that they really only reject the "all else being equal" (*ceteris paribus*) condition of these hypotheses, for as demonstrated in Figures 5C and 5D, the DRB1*15:01 allele is significantly correlated with PWHA versus NDs and PWHA/Inh+ versus PWHA/Inh− with tetrachoric correlation coefficients of 0.91 and 0.52, respectively ($P < .01$ for both).

3.6 | HLAcII isomer and FVIII domains also influence immunogenic potential

In Figure 6A, wherein we report the HLAcII-isomer effects, the DP category serves as the baseline/reference. Against this baseline, the DQ isomers presented significantly fewer FVIII-derived-peptides with a risk ratio of 0.60 (0.51, 0.71), whereas the DR

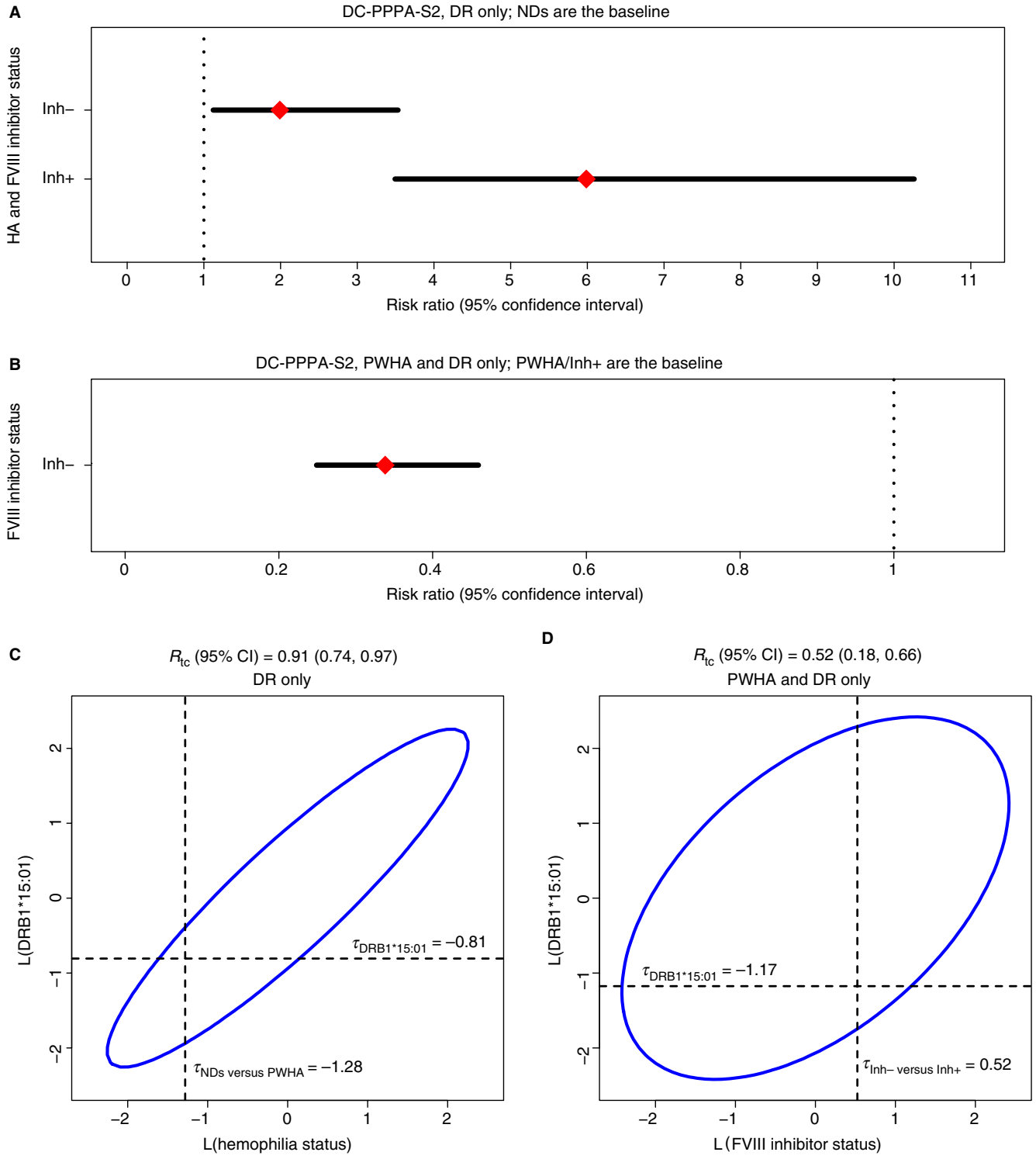


FIGURE 5 Effect of HA and inhibitor status on DC FVIII-peptide presentation. A, risk ratio (RR) estimates (red diamonds) and 95% confidence intervals (CIs) (black lines) obtained in Model 3 with NDs as baseline for PWHA positive and negative for inhibitors (Inh+ and Inh–). B, RR estimate & 95% CI obtained in Model 3 with PWHA/Inh+ as baseline for PWHA/Inh–. C, a tetrachoric correlation (R_{tc}) plot of DRB1*15:01 allele status (presence or absence) vs. HA status (NDs or PWHA) including the (1) underlying normally distributed liability functions, L(DRB1*15:01) and L(HA status), each with its associated threshold, denoted tau (τ), which are appropriately subscripted, and (2) 95% CI for the bivariate standard normal (blue ellipse). D, R_{tc} plot of the DRB1*15:01 allele status vs. inhibitor status (Inh+ or Inh–). FVIII, factor VIII; HA, hemophilia A; NDs, normal donors; PWHA, patients with HA

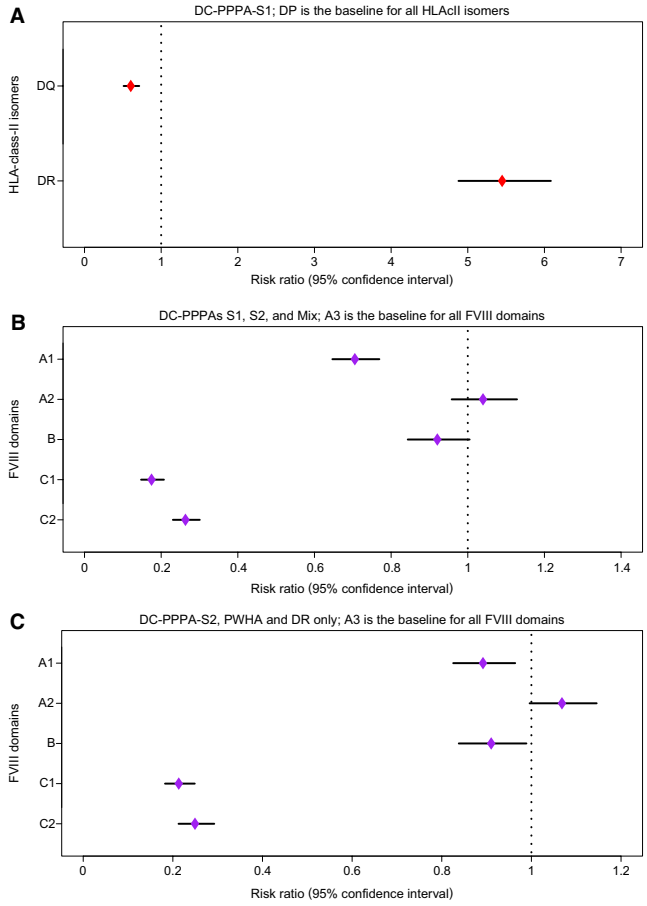


FIGURE 6 Effect of HLAcII isomers and FVIII domains on DC FVIII-peptide presentation. Risk ratios (red & purple diamonds) and 95% confidence intervals (black lines) for: A, HLAcII isomers (red diamonds) analyzed in Model 1 with DP as baseline for DQ and DR; B, FVIII domains (purple diamonds) analyzed in Model 2 with the A3 domain as baseline for the A1, A2, B, C1, and C2 domains; C, FVIII domains (purple diamonds) analyzed in Model 3 with A3 as baseline for A1, A2, B, C1, and C2. FVIII, factor VIII; HLAcII, human leukocyte antigen class II

isomers presented significantly more FVIII-derived-peptides with a risk ratio of 5.45 (4.88, 6.08). These experimental results demonstrate that for the immunogenic potential of FVIIIs, the DR allotypes comprise the most important HLAcII isomer by a wide margin.

In Figures 6B and 6C, we show the relative importance (to immunogenic potential) of the various domains in FVIIIs, which are ordered as follows: H_2N-A1-A2-B-A3-C1-C2-CO_2H. However, FVIIIs contain three acidic-residue-rich-connecting segments (a1, a2, and a3) oriented between the A-domains and B-domain as follows: H_2N-A1-a1-A2-a2-B-a3-A3-C1-C2-CO_2H. To simplify our analysis, IPSs extending into a1, a2, or a3 from the A1-domain, A2-domain, or B-domain connected to its N-terminus, respectively, were included with that domain, while IPSs with N-terminal residues in a1, a2, or a3 were included with the A2-domain, B-domain, or A3-domain, respectively, whether they extended into that domain or not. We compared the number of IPSs derived from each FVIII domain (augmented where relevant as just discussed). Initial examination of the data showed A3 to be numerically dominant over the other domains, making it the logical choice for baseline/reference. Figures 6B and 6C are consistent in that relative to the A3-domain, the A1, C1, and C2 domains all yielded significantly fewer HLAcII-bound/FVIII-derived-peptides. Also consistent between Figures 6B and 6C is that the A2-domain does not differ significantly from the A3-domain baseline. The only (slight) inconsistency is that the B-domain is not significantly different from the A3-domain in the former analysis (Figure 6B) but is significantly different from the baseline in the latter analysis (Figure 6C).

4 | DISCUSSION

We hypothesize that a patient's HLAcII repertoire plays the "gate keeper" role in determining whether a given FVIII will (considering the unique set of patient-specific, product-specific, and treatment-specific variables of immunogenicity risk in his genome and environment) activate proliferation/differentiation of his remaining naïve FVIII-specific CD4 T-cells (if any) into T_H-cells capable of inhibitor induction.[21-24,32,33] Thus, using DC-PPPA-S1, DC-PPPA-S2, and DC-PPPA-Mix, we measured the extent to which DCs from 28 NDs and 6 PWHA, 2 with and 4 without inhibitors (Inh+ and Inh−), presented peptides derived from 6 FVIIIs (some ± pdVWF and others only with or without pdVWF) in complexes with the distinct molecules comprising their individual HLAcII repertoires. Peyron et al.[31] recently presented DQ and DR findings from their DC-PPPA studies of nine NDs. Notably, via this manuscript and our companion manuscript by Jankowski et al.,[24] we are the first to report findings from studies of 1) all isomers comprising individual HLAcII repertoires (i.e., DP, DQ, and DR) and 2) PWHA, both with and without inhibitors (Inh+ and Inh−, respectively).

In Figure 2E we plotted the residue counts in the FVIII-derived-peptides eluted from the distinct allotypes of separately isolated 1) DP, DQ, and DR isomers from DC-PPPA-S1; and 2) DR isomers for DC-PPPA-S2. The asterisks placed at the top of the figure indicate the midpoint of ranges from the N-termini to C-termini of the IPSs (relative to the FL-FVIII amino acid-sequence) reported in other studies of potentially immunogenic peptides as well as the corresponding locations from the current study. The purple asterisks indicate peptides that were reported in van Haren et al. with residue midpoints after rounding to the nearest integer of 89, 466, and 733.[28,29] The green asterisk located at residue 376 represents a peptide in Hu et al., van Haren et al., and Sorvillo et al.[28-30,50] The study by Hu et al.[50] is of interest because they demonstrated in CD4 T-cell stimulation assays that their reported peptide, which spanned residues 371 to 400, consistently had the highest immunogenicity index in NDs, PWHA/Inh−, and PWHA/Inh+. The orange asterisk located at midpoint residue 1775 represents a peptide reported in Reding et al., van Haren et al., and Sorvillo et al.[28-30,51] Notably, Reding et al.[26] also demonstrated that peptides derived from the A3-domain were most frequently and strongly recognized by CD4 T-cells, which is

consistent with our finding that the A3-domain yielded significantly more HLAcII-presented peptides than the A1, B, C1, and C2 domains.

Our results shed light on the current controversy regarding the relative risks of pdFVIIIs versus rFVIIIs for the development of inhibitors, as discussed in recent reviews.[52-54] Data from the CANAL, RODIN, and EUHASS studies did not reveal a significant difference in the frequency of inhibitor development.[55-58] However, recent findings from the randomized clinical trial SIPPET demonstrated conclusively that rFVIIIs (i.e., that lack VWF) are associated with a significantly increased incidence of FVIII inhibitors compared to pdFVIIIs, which contain pdVWF.[14] Similar results were reported for a nationwide prospective study of Slovakian patients with severe HA.[59] Additionally, a recent study by Calvez et al. found a lower frequency of inhibitor development for a specific pdFVIII (Factane®) compared to two FL-rFVIIIs (Advate and Kogenate®).[60] Our results support the findings of Peyvandi et al., Batorova et al., and Calvez et al. only to the extent that the comparison is between pdFVIII + pdVWF and FL-rFVIII ± pdVWF in which the latter, that is, Advate, yielded significantly more HLAcII-bound/FVIII-derived-peptides even when it was administered to the same DCs after preincubation with the same molar excess (i.e., 13.4:1) of the same pdVWF.[14,59,61] We also found that both BDD-rFVIII$_4$ and the rFVIII mixture (Mix-rFVIII) yielded significantly more HLAcII-bound/FVIII-derived-peptides compared to pdFVIII + pdVWF.

When we compared pdFVIII + pdVWF to BDD-rFVIII$_1$ + pdVWF, or to BDD-rFVIII$_4$ + pdVWF, however, the results changed dramatically in that while there was no significant difference for the first comparison, the latter showed that BDD-rFVIII$_4$ + pdVWF yielded significantly fewer HLAcII-presented/FVIII-derived-peptides. This result is consistent with reports from several studies showing significant differences in the frequency of inhibitor development when comparing different rFVIIIs.[55,61-65] Indeed, it is plausible that these reported differences in regard to rFVIII concentrates were produced by differences in the extent to which they were presented by DCs to naïve FVIII-specific CD4 T-cells and (possibly) by B-cells to FVIII-specific T_H-cells.

It is well known that VWF modulates FVIII immunogenicity.[18,30,41,53,66,67] Our results speak to this issue. We showed that rFVIIIs coadministered with a pdVWF present significantly fewer HLAcII-bound/FVIII-derived-peptides than the same rFVIIIs alone. At the peptide level, this finding was first reported by Sorvillo et al., who demonstrated that VWF 1) by itself is not endocytosed by DCs; 2) is internalized by DCs only when coadministered with rFVIIIs; and, most importantly, 3) when coadministered with a rFVIII noticeably decreases HLAcII-presentation of FVIII-derived-peptides.[30] Our results and those of Sorvillo et al.[30] together confirm the hypothesis proposed by Dasgupta et al.[18] that VWF reduces FVIII immunogenicity by restricting its uptake by APCs.

Under our Gate Keeper Hypothesis, a requirement for the development of inhibitors in a PWHA is the ability of his HLAcII molecules to bind tightly FVIII-derived-peptides generated by his 1) DCs to present to naïve FVIII-specific CD4 T-cells, which—via immune synapse formation with these APCs—receive critical signals to become T_H-cells; and 2) FVIII-specific B-cells to present to the FVIII-specific T_H-cells, which—via immune synapse formation—provide the signals these B-cells need to become plasma cells that secrete neutralizing anti-FVIII antibodies. For this reason, a straightforward candidate for genetic association analysis of inhibitor pathogenesis (and arguably the earliest investigated candidate) has been the highly polymorphic genes encoding HLAcII molecules.[9] The haplotype DQB1*06:02/DRB1*15:01 was associated with inhibitors in three studies.[7,9,10] Additionally, the DRB1*15:01 allele was significantly and suggestively associated with inhibitors by Nathalang et al.[8] and Wieland et al.,[68] respectively. Notably, DRB1*15:01 has also been implicated in the pathogenesis of drug-induced diseases and autoimmunity.[69-72] Our results are consistent with the established role of DRB1*15:01 in FVIII immunogenicity, autoimmune diseases, and adverse drug responses in that the DQB1*06:02/DRB1*15:01 haplotype was associated with significantly greater IP.

Peyron et al. recently reported results from a DC-PPPA-based study that compared the FVIII-derived peptides bound to the DQ isomers versus the DR isomers of the HLAcII repertoires in nine unrelated NDs.[31] While the DQ allotypes contributed to HLAcII presentation, they presented significantly fewer FVIII-derived peptides than DR. We observed the same result here but also found that DQ allotypes presented significantly fewer FVIII-derived peptides than both DP and DR molecules. Recent results from the organ, tissue, and cell transplantation literature, however, caution against downplaying the influence that DQ and DP may exert in inhibitor development.[73] Specifically, although DR molecules are the chief HLAcII-based determinant of graft rejection and graft-versus-host-disease, DP and DQ isomers contribute significantly to the development of adverse transplant outcomes.[73,74] Thus, when these findings are considered with the results presented here and in our companion manuscript,[24] it appears that it is necessary to collect data on the entire HLAcII repertoire to understand FVIII immunogenicity fully.

In summary, our findings from the DC-PPPAs and MAPPs analyses performed herein demonstrate its utility for identifying and characterizing the molecular determinants of FVIII immunogenicity. Consistent with our Gate Keeper Hypothesis,[23] we have shown that significant HLAcII/FVIII-peptidome level differences likely mediate the distinct risks of inhibitor development observed clinically for various FVIIIs including 1) pdFVIIIs versus rFVIIIs; 2) non-engineered versus engineered rFVIIIs; and 3) next-generation-approved versus original-FDA-approved rFVIIIs.[34-39,75] We showed that preincubation of FVIIIs with pdVWF significantly decreases their HLAcII-bound/FVIII-derived-peptide counts, and that this protective effect is differentially manifested across rFVIIIs. For example, whereas the pdVWF protective effect was insufficient to equalize the immunogenicity potential of FL-rFVIII to that of pdFVIII, there was a dramatic shift in the immunogenicity potential of BDD-rFVIII$_4$ relative to that of pdFVIII from being significantly greater without pdVWF to being significantly less with pdVWF. We found that the DQB1*06:02/DRB1*15:01 haplotype was associated with significantly greater peptide presentation than the other DQB1/DRB1 haplotypes. This is not surprising given that it is an established

risk factor for multiple autoimmune diseases and the development of anti-(protein-drug)-antibodies including inhibitors. While we also found that all isomeric types of HLAcII molecules present FVIII-derived-peptides, they do so at significantly different levels: DR > DP > DQ. Finally, in agreement with published findings, we found significantly more HLAcII-presented/FVIII-derived peptides from the A2 and A3 domains than from the other domains. Despite the power of this approach, there are some limitations worthy of mention including the current inability of (i) the DC-PPPA to test therapeutic proteins within the complex fluids they are sampled from *in vivo* (i.e., plasma); (ii) the MS strategy employed to establish absolute (and thus) relative peptide counts without the use of known quantities of spiked-in isotope-labeled reference peptides; and (iii) the existing proteomics tools to identify peptides with co-translational/post-translational modifications (e.g., N-linked glycans). An additional limitation is that the FVIII-derived-peptide counts across the three DC-PPPAs are not normalized to the HLAcII-presented endogenous peptides. Nevertheless, by anchoring our analytic perspective to the gate keeper HLAcII molecules, MAPPs analysis has the potential to improve our ability to predict and prevent inhibitor development.

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CONFLICT OF INTEREST

M. Hofmann, J. Powell, H. Huynh, A. Verhagen,' and E. Maraskovsky are employees of CSL Behring; P. Lehmann is the Founder and CEO of Cellular Technology Limited.; B. Luu and L. Dinh are employees of Haplogenics Corporation; and T. Howard is the CSO for Haplogenics Corporation. The findings and conclusions in this article have not been formally disseminated by the Food and Drug Administration and should not be construed as representing any agency determination or policy.

AUTHOR CONTRIBUTIONS

V. P. Diego designed the statistical analysis plan for the FED Study, performed all statistical analyses of the study data, and both drafted and edited the manuscript. B. W. Luu designed and maintained the FED Study database, performed bioinformatic analyses of study data, created figures and tables of the data, and helped in the drafting, review, and editing of the manuscript. M. Hofmann generated and characterized all FVIII preparations for analysis by the DC-PPPAs and participated in the design of experiments as well as in the review and editing of the manuscript. L. V. Dinh collected, processed, stored, and shipped the FVIIIs used in the DC-PPPAs and helped in the drafting, review, and editing of the manuscript. M. Almeida performed computational bioinformatics analyses of study data, created figures, and helped in the review and editing of the manuscript. J. S. Powell facilitated communications between the collaborative research teams and helped in the review and editing of the manuscript. R. Rajalingam interpreted the HLAcII allelic, genotypic, allotypic, and haplotypic data, and both reviewed and edited the manuscript. R. Kellerman and Y. Park carried out the human subjects research components of this study including the acquisition of IRB approval at the UNC-Chapel Hill, the recruitment, consent, and enrollment of patients, as well as the collection, processing, and shipping of the PBMC samples. Z. E. Sauna participated in the design of experiments and reviewed the manuscript. E. Maraskovsky participated in the design of experiments as well as in the review and editing of the manuscript. N. S. Key designed the clinical aspects of the study including the collection of PBMC samples from hemophilia patients by leukopheresis and reviewed the manuscript. H. Huynh and A. M. Verhagen performed experiments to evaluate internalization of the FVIIIs by DCs obtained from three NDs. J. M. Peralta, S. Kumar, J. Curran, S. Williams-Blangero, P. V. Lehmann, M. A. Escobar, and J. Blangero analyzed and interpreted study data, and both reviewed and edited the manuscript. T. E. Howard designed the research plan, oversaw the project, and wrote the manuscript.

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SUPPORTING INFORMATION

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

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