FVIII peptides presented on HLA-DP and identification of an A3 domain peptide binding with high affinity to the commonly expressed HLA-DP4

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Abstract

The development of neutralizing antibodies (inhibitors) against coagulation factor VIII (FVIII) poses a major challenge in hemophilia A (HA) treatment. The formation of FVIII inhibitors is a CD4$^+$ T-cell-dependent mechanism which includes antigen presenting cells (APC), B- and T-helper lymphocytes. APC present FVIII-derived peptides on major histocompatibility complex class II (MHC-II) to CD4$^+$ T cells. We previously established a mass spectrometry-based approach to delineate the FVIII repertoire presented on HLA-DR and HLA-DQ. In this study, specific attention was directed towards the identification of FVIII peptides presented on HLA-DP. A data-set of naturally processed FVIII peptides was generated by incubating human FVIII with immature monocyte-derived dendritic cells (moDC) from HLA-typed healthy donors. Using this method, we identified 176 to 1,352 different HLA-DP presented peptides per donor, including 26 different FVIII-derived peptides. The most frequently presented peptides derived from the A3 and C2 domains of FVIII. Comparison of the FVIII repertoire presented on HLA-DP with that presented on HLA-DR revealed considerable overlap but also suggested preferential presentation of specific peptides on either HLA-DR or HLA-DP. Fourteen FVIII peptides presented on HLA-DP were synthesized and evaluated for their binding ability to the commonly expressed HLA-DP4 molecule which is highly prevalent in the Caucasian population. Peptide binding studies showed that 7 of 14 peptides competed with a reference peptide to HLA-DP4. Interestingly, an A3 domain-derived peptide bound with high affinity to HLA-DP4, positioning this peptide as a prime candidate for the development of novel peptide-based tolerogenic strategies for FVIII inhibitors.

Introduction

Hemophilia A is an X-linked recessive disease caused by mutations in the gene encoding coagulation factor VIII (FVIII). The lack of functional endogenous FVIII in individuals with severe hemophilia A can cause spontaneous and life-threatening hemorrhages. The gold standard in preventing or treating hemorrhages is the administration of plasma-derived or recombinant FVIII.[1] The main complication of the FVIII replacement therapy is the development of neutralizing anti-FVIII antibodies, commonly known as 'inhibitors', in a substantial fraction of hemophilia A patients.[2-4] So far, the only effective therapy for the eradication of inhibitors is immune tolerance induction (ITI), which involves the intravenous administration of high doses of FVIII over a period of weeks to years to induce antigen-specific tolerance. In addition to its high costs, the success rate for ITI varies from 60% to 80%.[2,3] Over the past years, non-factor replacement therapies have been developed which include the chimeric bispecific humanized antibody emicizumab, the anti-tissue factor pathway inhibitor (TFPI) humanized monoclonal antibody concizumab, and an RNAi targeting antithrombin Fitusiran.[5] Emicizumab is now widely used for treatment of hemophilia A patients with and without inhibitors.[6] Although new therapeutic products are now available, breakthrough bleeds in patients

with hemophilia A are still treated with FVIII, and hence there is an urgent need to develop strategies to induce and maintain tolerance.[7] FVIII-specific antibodies isolated from patients with inhibitors in hemophilia A primarily target the A2, A3, and C2 domains of FVIII.[4,8] These antibodies mostly belong to the IgG1 and IgG4 subclasses, suggesting the involvement of CD4+ T cells in the immune response against FVIII.[8] The development of inhibitors is a complex process that includes lack or loss of central and peripheral immune tolerance.[9] Dendritic cells (DC) play a crucial role as antigen-presenting cells (APC) in the immune response to FVIII. DC take up FVIII antigens through various endocytic processes and present FVIII-derived peptides on MHC class II molecules. Mature DC express co-stimulatory molecules and interact with CD4+ T cells, leading to their activation. Activated CD4+ T cells stimulate B cells, resulting in the production of FVIII-specific memory B cells and plasma cells that secrete anti-FVIII antibodies. Memory B cells are mostly found in the spleen or bone marrow and can differentiate into plasma cells upon re-exposure to FVIII.[10,11] Immunodominant T-cell epitopes that trigger inhibitor development in hemophilia A patients derive from the repertoire of FVIII peptides displayed on MHC class II. The identification and removal of immunodominant epitopes from FVIII sequence can potentially be employed for the development of less immunogenic biotherapeutics.[12,13] Alternatively, FVIII immunogenic peptides can be used to induce tolerance employing peptide-containing tolerogenic vaccines[14] or peptide-loaded rapamycin containing nanoparticles.[15]

MHC class II molecules are encoded by the classical human leukocytes antigen (HLA)-DR, HLA-DQ and HLA-DP alleles and the non-classical MHC II molecules HLA-DM and HLA-DO on chromosome 6.[16] To date, naturally FVIII-derived peptides presented on HLA-DR and HLA-DQ have been extensively characterized.[17-21] In parallel approaches, patient-derived CD4+ T cells recognizing specific FVIII peptides have been identified in hemophilia A patients with inhibitors.[21-29] Additionally, FVIII-specific CD4+ T cells and FVIII-specific regulatory T cells (Treg) have been described in healthy donors.[21,30] FVIII peptide presentation on HLA-DP has so far only been addressed in a few studies.[21,31] The 16 HLA-DPA1 and 118 HLA-DPB1 alleles form a variety of haplotypes. Nevertheless, only the haplotypes HLA-DPA1*0103/DPB1*0401 and HLA-DPA1*0103/DPB1*0402 are commonly found across global populations.[32] These specific haplotypes together exhibit a gene frequency of approximately 50% in Europe, 60% in South America, 80% in North America, 60% in India, 25% in Africa, and 15% in Japan;[32] consequently, they are present in around 76% of Caucasians.[32] Therefore, CD4+ T-cell epitopes restricted to HLA-DP4 are expected to be recognized by a large portion of individuals. The primary goal of this research was to explore the naturally occurring FVIII peptidome presented on HLA-DP using mass spectrometry-based immunopeptidomics, and to compare it to the repertoire presented on HLA-DR. The ultimate objective was to select potential HLA-DP4-presented peptides that can be used for the development of peptide-based approaches for the induction of tolerance to FVIII.

Methods

Subjects

Buffycoats from HLA-typed healthy volunteers were collected in accordance with Dutch regulations and following approval from Sanquin Ethical Advisory Board in accordance with the Declaration of Helsinki.

Purification and elution of HLA-DP-presented peptides on monocyte-derived dendritic cells

Factor VIII-loaded mature monocyte-derived dendritic cells (moDC) were harvested and resuspended in 500 μL of lysis buffer (10 mM Tris-HCl pH 8.0, 0.25% octyl-β-D-glucopyranoside, 1% sodium deoxycholate and 10 mM EDTA) for 30 minutes (min) at 4°C. Lysates were centrifuged at 20,000 x g for 15 min at 4°C and supernatants were incubated with 300 μL of Sepharose beads containing the anti-HLA-DP mAb B7-21 or anti-HLA-DR mAb L243. Following overnight incubation at 4°C, Sepharose beads were washed twice with lysis buffer, and five times with 10 mM Tris-HCl pH 8.0. Bound MHCII molecules were eluted by incubation with 10% acetic acid for 10 min at room temperature. Eluates were collected and heated for 15 min at 70°C to dissociate peptide/MHCII complexes, and analyzed using mass spectrometry (*Online Supplementary Methods*).

HLA-DP4 binding assay

Quantitative peptide-MHCII competition binding assays were performed to determine binding affinities of FVIII peptides to HLA-DP4, essentially as described.[32,33] Varying concentrations (0-30 μM) of non-biotinylated FVIII competitor peptides were incubated overnight at 37°C with constant concentrations of DPA1*0103/DPB1*0401 (5 nM) and 10 nM of a biotinylated leucyl-cystinyl aminopeptidase derived peptide (KKYFAATQFEPLA; residues 286-298; UniProt entry Q9UIQ6) in phosphate buffer (100 mM phosphate buffer, 2.5 mg/mL octyl-β-D-glucopyranoside, pH 5.9). The formed DPA1*0103/DPB1*0401/peptide complexes were captured by incubation for 2 hours (hr) at 37°C in a 96-well microtiter plate (MaxiSorb, Nunc) precoated with 50 μL of 5 μg/mL of anti-HLA class II antibody, clone IVA12, in PBS (pH 7.2) and blocked with 5% FCS in PBS. Unbound complexes and free peptide were removed from the plate by washing with 0.1% Tween-20 in PBS. Streptavidin-HRP (0.2 μg/mL in PBS 0.1% Tween20) was added to each well of the plate and incubated for 1 hr at 2-8°C. After washing the plate, substrate solution, TMB (3,3',5,5' tetramethylbenzidine) (100 μL/well), was added, and the plate was incubated at

room temperature for 15 min. ELISA stopping solution (0.5M H_2SO_4) was added and absorbance at 450 nm measured on a FLUOstar OPTIMA fluorometer (BMG Labtech, Ortenberg, Germany). Sigmoidal binding curves were simulated and IC_{50} values calculated for the FVIII peptides, based on their competition with the reference peptide. Data were reported as IC_{50} values. Peptides with IC_{50} values below 1 μM were considered to bind with high affinity.

Statistical analysis

Peptides were identified using Proteome Discoverer 2.4 (Thermo Scientific). Raw Xcalibur data files were screened against the UniprotKB reviewed .H_sapiensdatabase with a mass deviation of 20 ppm, a fragment mass tolerance of 0.8 Da, and a false positive discovery rate of 95%. All identified FVIII-derived peptides with high and medium confidence were grouped and aligned for each donor.

Results

The repertoire of factor VIII-derived peptides presented on HLA-DP

To explore the FVIII peptides presented on HLA-DP, we employed moDC from 14 HLA-typed healthy donors. Immature moDC were pulsed with 100 nM of full-length FVIII and maturated overnight with lipopolysaccharide to increase the expression of HLA-peptide complexes on the cell surface. Mature moDC were lysed and HLA-DP molecules were purified using monoclonal antibody B7-21 coupled to CNBr Sepharose 4B. Peptides bound to purified HLA-DP were eluted, detected by mass spectrometry and analyzed using Proteome Discoverer as described in the experimental procedures. Using this approach, we identified approximately 1,000 HLA-DP-associated peptides from 7 donors. A combined list of proteins from all 14 donors was generated to perform a functional enrichment analysis. The data source includes annotation from three gene ontology (GO) subsets: cellular compartment (*Online Supplementary Figure S1A*), biological process (*Online Supplementary Figure S1B*), and molecular function (*Online Supplementary Figure S1C*). Enrichment analysis revealed terms with the highest score with emphasis on the adjusted *P* values as a prominent scoring metric (*Online Supplementary Figure S1A*). Our findings show that cell adhesion molecules and other protein binding complexes are commonly presented on HLA-DP (*Online Supplementary Figure S1C*). In accordance with previous findings our data further confirm that many peptides are derived from proteins involved in the MHC class II pathways and, more broadly, in the "immune system process"[18] (*Online Supplementary Figure S1B*). These findings highlight that, similar to HLA-DR and HLA-DQ peptides, which result from the processing of endogenous proteins located in various cellular compartments within antigen-presenting cells, HLA-DP also efficiently presents these peptides.

We assessed the repertoire of FVIII peptides presented on HLA-DP for 14 donors. For 10 out of 14 donors, immature DC were incubated in the presence and absence of FVIII (donors E, F, G, H, I, J, K, L, M and N). Immature DC derived from 4 donors were incubated with FVIII only (donors A, B, C and D). The amount of HLA-DP-presented peptides in the analyzed donors ranged from 176 to 1,352 distinct peptides (Table 1). As expected, FVIII-derived peptides were presented only on FVIII-pulsed moDC. The unique peptides attributed to FVIII ranged from 2 to 41 per donor (Table 2). The detection of different peptides with the same core peptide sequence is explained by amino and carboxy-terminal trimming of MHC class II bound peptides which does not affect the core peptide bound to residues within the MHC peptide binding groove.[34] Table 2 shows the complete list of FVIII peptides for each donor. Our results indicate that multiple FVIII-derived peptides are presented on HLA-DP. Specifically, the most prevalent peptides originate from the A3 and C2 domains. Approximately, half of the donors presented peptides from the A1, *a1*, and A2 domains. In contrast, peptides from the *a3*, B and C1 domains were less common, with each being found only in 2 or 3 donors. Interestingly, 2 of the FVIII peptides found were localized in the *a1* and *a2* region. The *a1* region-derived peptide overlapped with the thrombin cleavage at Arg391. The peptide derived from the *a2* region corresponded to the carboxy-terminus of the FVIII heavy chain. Altogether, these data suggest that HLA-DP molecules preferentially present a distinct set of peptides mostly derived from the A3 and C2 domains of FVIII.

Peptides presented on HLA-DP and HLA-DR

The repertoire of FVIII-derived peptides presented on HLA-DP and HLA-DR was compared for 11 donors (donors A, B, C, D, H, I, J, K, L, M and N). The total number of unique peptides presented on HLA-DR ranged from 339 to 2,467 (Table 1), consistently exceeding that observed for HLA-DP. Similarly, the number of FVIII peptides presented on HLA-DR was higher than those derived from HLA-DP. Upon pooling the HLA-DR and HLA-DP peptide sequences from the 11 donors, we identified a total of over 16,000 HLA-DR-presented peptides and over 9,000 HLA-DP-presented peptides. A detailed comparison of the peptide sequences presented on HLA-DR and HLA-DP for each donor revealed only a partial overlap between the two sets. Specifically, we found that approximately 20% of the HLA-DP sequences were also presented on HLA-DR, and around 8% of HLA-DR peptides were also presented on HLA-DP. This indicates a certain degree of redundancy but also highlights the unique repertoire associated with HLA-DR and HLA-DP. With an average length of 16 amino acids, the peptides presented by both HLA-DP and HLA-DR were similar in size (*data not shown*). Overall, these findings suggest that while there is some overlap, HLA-DR

Table 1. The repertoire of naturally presented peptides on HLA-DP.

Donors	HLA DRB1*	HLA DPB1*	HLA-DRB1*			HLA-DPB1*			HLA-DRB1*			HLA-DPB1*		
			No FVIII			No FVIII			FVIII			FVIII		
	Alleles	Alleles	Prot.	Pep.	F8 pep.	Prot.	Pep.	F8 pep.	Prot.	Pep.	F8 pep.	Prot.	Pep.	F8 pep.
A	03:01 15:01	04:01 04:01	-	-	-	-	-	-	285	1105	36	165	368	10
B	03:01 03:01	02:01 04:01	-	-	-	-	-	-	363	1049	2	148	262	3
C	03:01 07:01	04:01 19:01	-	-	-	-	-	-	292	974	13	213	470	5
D	03:01 07:01	04:01 - 09:01	-	-	-	-	-	-	214	339	8	169	410	9
E	-	04:AJET 04:AJEU	-	-	-	238	462	ND	-	-	-	219	474	29
F	-	03:01 04:01	-	-	-	55	135	ND	-	-	-	120	221	4
G	-	04:01 04:FNVS	-	-	-	372	1063	ND	-	-	-	328	970	41
H	04:01 15:01	04:01 04:01	411	1892	ND	244	811	ND	435	2165	63	304	834	27
I	03:01 13:01	04:01 04:01	267	995	ND	135	430	ND	281	1026	23	135	335	14
J	10:01 10:01	04:01 04:01	562	2301	ND	441	1146	ND	559	2467	51	454	1219	26
K	04:01 15:01	04:01 04:01	386	1712	ND	373	982	ND	487	2207	73	471	1328	41
L	03:01 15:01	04:01 04:01	-	-	-	-	-	-	325	1378	27	95	176	2
M	03:01 15:01	04:01 04:01	213	794	ND	344	848	ND	346	1520	15	386	980	4
N	04:04 15:01	04:01 04:01	-	-	-	422	1172	ND	512	2436	17	491	1352	5

Fourteen genotyped donors were used for the immunopeptidomics analysis. The donors were analyzed under varying conditions. For 11 donors (A, B, C, D, H, I, J, K, L, M, N), the repertoire of peptides presented on HLA-DP was compared to that on HLA-DR. For 9 donors (E, F, G, H, I, J, K, M, N), we compared the antigen presented on HLA-DP of no factor VIII (FVIII) and FVIII-pulsed monocyte-derived dendritic cells. The tables include genotype information, the number of proteins (Prot.), unique peptides (Pep.), and FVIII peptides (F8 pep.) detected for each donor. For ambiguous genotypes, we used the MAC Service 2.19.0 from the National Marrow Donor Program to decode allele strings (*https://hml.nmdp.org/MacUI/*): HLA-DPB1*04:AJET corresponds to DPB1*04:01/23:01/33:01/51:01; HLADPB1*04:AJEU corresponds to DPB1*02:01/04:02/71:01/81:01; HLA-DPB1*04:FNVS corresponds to B*04:02/B*105:01.

molecules present a broader and more diverse array of peptides compared to HLA-DP.

The repertoire of factor VIII-derived peptides presented on HLA-DP and HLA-DR

The unique peptides attributed to FVIII presented on HLA-DR ranged from 2 to 73. They were grouped in 29 nested sets, which partially overlapped with the nested sets on HLA-DP (Table 3). Nineteen out of 26 nested sets presented on HLA-DP were also presented on HLA-DR. Despite the overlap, the number of donors presenting specific FVIII peptides differs between HLA-DP and HLA-DR (see Table 3). The HLA-DP nested sets were further compared with the previously studied HLA-DR sets.[20,31] Except for peptides 491-508 and 1959-1985, the analysis revealed that all the HLA-DP peptides were also found on HLA-DR and HLA-DQ molecules, confirming their nature as promiscuously presented peptides. To further compare the repertoire of FVIII peptides on HLA-DP and HLA-DR, the binding core of the unique FVIII-derived peptides was determined using NetMHCIIpan4.3. The core peptides were predicted with respect to the HLA haplotypes of the 14 donors. FVIII peptides presented on both HLA-DR and HLA-DP were predicted to bind with the same core peptide sequences to both MHC class II molecules. A few peptides were exclusively presented on HLA-DR or HLA-DP, suggesting that these core peptides are uniquely bound to either HLA-DR or HLA-DP (*Online Supplementary Table S1*).

Identification of peptide binding motifs to HLA-DP401

Eight of the donors included in this study were homozygous for HLA-DPB1*0401. This allowed us to assess the binding motif for this frequently occurring HLA-DP allele. To investigate the binding motifs of peptides to HLA-DP401, we

employed the Gibbs clustering algorithm (*Online Supplementary Figure S3*). This method is particularly suited for identifying motifs within peptide sequences by iteratively optimizing cluster assignments and motif alignments. As input for the Gibbs clustering, we provided the list of naturally presented peptides from donors A, H, I, J, K, L, M and N, all of whom are homozygous for HLA-DPB1*04:01. This ensured that the resulting binding motifs are specific for HLA-DPB1*04:01. The Gibbs clustering algorithm was run multiple times to create 5 clusters. Out of the 5 clusters obtained, cluster 2 scored the highest Kullback-Leibler Distance (KLD) (*Online Supplementary Figure S3, left*). Subsequently, logo plots were generated for the two groups present in cluster 2. Group 1 of cluster 2 showed bit scores up to 0.4, but the resulting motif did not align with that reported by Castelli *et al*. and Falk *et al.*, showing weak conservation at the amino acid positions corresponding to the anchor residues.[32,35] In contrast, group 2 of cluster 2 showed stronger residue conservation, with bit scores reaching 0.6. In this group, phenylalanine (F) residues were

Table 2. Complete list of factor VIII peptides on HLA-DP.

A			DONORS													
F8	**Start**	**Peptides**	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**
A1	62	SVVYKKTLFVEFTDH														
	69	LFVEFTDHLFNIAKPRPP														
		FVEFTDHLFNIAKPR														
		FVEFTDHLFNIAKPRP														
		FVEFTDHLFNIAKPRPP														
		VEFTDHLFNIAKPRPP														
		VEFTDHLFNIAKPRPPW														
		EFTDHLFNIAKPR														
		EFTDHLFNIAKPRP														
		EFTDHLFNIAKPRPP														
	98	EVYDTVVITLKNMASHPVS														
		VYDTVVITLKNMASHPVS														
		YDTVVITLKNMASHPV														
		YDTVVITLKNMASHPVS														
		DTVVITLKNMASHP														
		DTVVITLKNMASHPV														
		DTVVITLKNMASHPVS														
a1	367	DDLTDSEMDVVRF														
	380	DDDNSPSFIQIRSVAKKHPK														
		DDNSPSFIQIRSVAKKHPK														
		NSPSFIQIRSVAKKHPK														
		SPSFIQIRSVAKKHPK														
A2	392	SVAKKHPKTWVHYIAAEEEDWDYAPLVLAPDDR														
		SVAKKHPKTWVHYIAAEEEDWDYAPLVLAPDDRSY														
		SVAKKHPKTWVHYIAAEEEDWDYAPLVLAPDDRSYK														
		KKHPKTWVHYIAAEEEDW														
		KHPKTWVHYIAAEEEDW														
	474	GEVGDTLLIIFKNQASRPYN														
		EVGDTLLIIFKNQASRPYN														
		VGDTLLIIFKNQASRPYN														
	491	PYNIYPHGITDVRPLYSR														
		YPHGITDVRPLYSR														
a2	743	EDISAYLLSKNNAIEPR														
		DISAYLLSKNNAIEP														
		DISAYLLSKNNAIEPR														
		ISAYLLSKNNAIEPR														
B	1131	GPSPKQLVSLGPEKSVEG														
		GPSPKQLVSLGPEKSVEGQ														
	1151	LGPEKSVEGQNFLSEKNK														
		GPEKSVEGQNFLSEKNK														
	1238	GTKNFMKNLFLLSTRQN														

Continued on following page.

B			DONORS													
F8	**Start**	**Peptides**	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**
a3	1668	EITRTTLQSDQEEID														
A3	1706	SPRSFQKKTRHYFIAA														
		SFQKKTRHYFIAAVERLWDYGMSSSPHVL														
		SFQKKTRHYFIAAVERLWDYGMSSSPHVLRN														
		KKTRHYFIAAVER														
		KTRHYFIAAVERLWD														
		AAVERLWDYGMSSSPH														
		VERLWDYGMSSSPH														
	1728	YGMSSSPHVLRN														
	1739	NRAQSGSVPQFKKVVFQEFTD														
		AQSGSVPQFKKVVFQEFTD														
	1784	AEVEDNIMVTFRNQASRPY														
		AEVEDNIMVTFRNQASRPYS														
		EVEDNIMVTFRNQASRP														
		EVEDNIMVTFRNQASRPY														
		EVEDNIMVTFRNQASRPYS														
		VEDNIMVTFRNQASRPY														
		VEDNIMVTFRNQASRPYS														
		EDNIMVTFRNQASRPY														
		DNIMVTFRNQASRPY														
		DNIMVTFRNQASRPYS														
	1820	EPRKNFVKPNETKTYFWK														
		EPRKNFVKPNETKTYFWKVQ														
	1959	IRWYLLSMGSNENIHSIHFSGHVFTVR														
	1984	VRKKEEYKMALYNLYPG														
		VRKKEEYKMALYNLYPGVF														
		VRKKEEYKMALYNLYPGVFE														
		RKKEEYKMALYNLYPG														
		RKKEEYKMALYNLYPGVF														
		RKKEEYKMALYNLYPGVFE														
		KKEEYKMALYNLYP														
		KKEEYKMALYNLYPG														
		KKEEYKMALYNLYPGV														
		KKEEYKMALYNLYPGVF														
		KEEYKMALYNLYPG														
		YPGVFETVEMLPSK														
		YPGVFETVEMLPSKA														
		YPGVFETVEMLPSKAG														
		YPGVFETVEMLPSKAGI														
		YPGVFETVEMLPSKAGIW														
C1	2095	LAPMIIHGIKTQGARQK														
		APMIIHGIKTQGARQ														
		APMIIHGIKTQGARQK														
	2117	ISQFIIMYSLDGKK														
C2	2199	MESKAISDAQITASSY														
	2223	SPSKARLHLQGRSNAWRPQ														
	2242	VNNPKEWLQVDFQKT														
		VNNPKEWLQVDFQKTM														
		VNNPKEWLQVDFQKTMK														
		VNNPKEWLQVDFQKTMKVT														
		VNNPKEWLQVDFQKTMKVTG														
		NNPKEWLQVDFQKT														
		NPKEWLQVDFQKT														
		NPKEWLQVDFQKTM														
		NPKEWLQVDFQKTMK														

Continued on following page.

B			DONORS													
F8	**Start**	**Peptides**	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**
		NNPKEWLQVDFQKTMKVT							■							
		NPKEWLQVDFQKT	■	■	■	■	■	■								
		NPKEWLQVDFQKTM	■						■							
		NPKEWLQVDFQKTMK	■				■		■							
		NPKEWLQVDFQKTMKV					■		■							
		NPKEWLQVDFQKTMKVT					■		■							
		NPKEWLQVDFQKTMKVTG							■							
	2271	LTSMYVKEFLISSSQ					■									
		LTSMYVKEFLISSSQDG					■									
		SMYVKEFLISSSQ					■									
		YVKEFLISSSQDGHQW											■			

Factor VIII-derived peptides on HLA-DP were identified using a mass spectrometry-based approach. The complete list of FVIII peptides from 14 donors sorted on their sequence localization is displayed. FVIII peptides belonging to the (A) heavy (A1-*a1*-A2-*a2*-B) and (B) light (*a3*-A3-C1-C2) chains are shown. Each column represents the results for an individual donor. Peptides identified for each donor are depicted in gray..

observed in positions 2 and 7 (*Online Supplementary Figure S3, right*). While these residues were expected in positions 1 and 6,[32,36] the distance between the two phenylalanine residues remains consistent with what was anticipated, suggesting that the overall binding motif is preserved despite the positional shift. The observed shift in residue position may be attributed to the inherent flexibility of the Gibbs sampling algorithm in optimizing sequence alignment and/or the lack of a strong C terminal anchor residue (M. Nielsen, personal communication, 2024).

Binding affinity of factor VIII peptides to HLA-DP401

The dataset of FVIII-derived peptides presented on HLA-DP was organized into 26 nested sets. Fourteen out of 26 peptides were tested for their ability to bind to the commonly expressed HLA-DP401 molecule which is encoded by DPA1*0103/DPB1*0401 alleles. The selected peptides were then tested in a binding assay where they competed with a reference peptide derived from leucyl-cystinyl aminopeptidase which is known to bind with high affinity to the HLA-DP401 molecule.[32,35] The percentage inhibition (% inhibition) was calculated, and the binding affinity was quantified as IC_{50} values. Peptides with high affinity inhibited the binding of the reference peptide at very low concentrations, while those with lower affinity required higher concentrations (Figure 1A). Seven peptides showed no inhibition of the reference peptide binding to HLA-DP401. The IC_{50} values for each peptide are provided in Figure 1B. Among the peptides tested, the P10 peptide, which is derived from residues 1984-2015 in the A3 domain, exhibited the highest affinity for binding to the HLA-DP401 molecule. Similarly, peptide P1 exhibited notable affinity for HLA-DP401. However, the majority of the peptides exhibited either lower affinity or failed to bind (*Online Supplementary Figure S2*). The observed high affinity binding of the P10 peptide, which was found in 12 out of 14 donors suggests preferential presentation of peptides from this specific region of FVIII on HLA-DP401.

Discussion

The development of inhibitory antibodies against exogenous FVIII poses a significant challenge in the treatment of hemophilia A.[36] A comprehensive understanding of the immunogenicity of FVIII holds the potential to drive the development of novel strategies aimed at the induction and maintenance of FVIII tolerance. In this regard, epitope-specific immunotherapy has already been designed for allergic and autoimmune diseases,[37] such as Graves disease,[38] rheumatoid arthritis,[39,40] multiple sclerosis,[41] and celiac disease.[42] Recently, Pletincxk and co-workers[14] identified antigen-processing-independent epitopes (apitopes) from immunodominant T-cell FVIII epitopes which selectively bind steady-state DC in lymphoid organs and do not bind B cells or monocytes *in vivo*. Peptide-receptive MHC class II molecules are abundantly expressed on the surface of steady-state and immature DC, which are known to induce tolerance *in vivo*. Maldonado *et al.*[15] reported that nanoparticles encapsulating rapamycin and a mixture of MHC class II-binding peptides induced specific tolerance to co-administered FVIII. Zhang *et al.*[43] employed a similar methodology that involved encapsulating the complete FVIII protein, rather than selected MHC class II-binding peptides. This approach was selected to tackle the inherent MHC class II heterogeneity. The investigation of FVIII peptidome presented on MHC class II molecules offers valuable prospects in identifying suitable candidates for advancing and optimizing peptide-based therapeutic strategies. To date, extensive research has been conducted on the FVIII peptidome presented on HLA-DR and HLA-DQ.[18-20,31] However, limited attention has been given to the

Table 3. Comparison of factor VIII peptides on HLA-DP and HLA-DR.

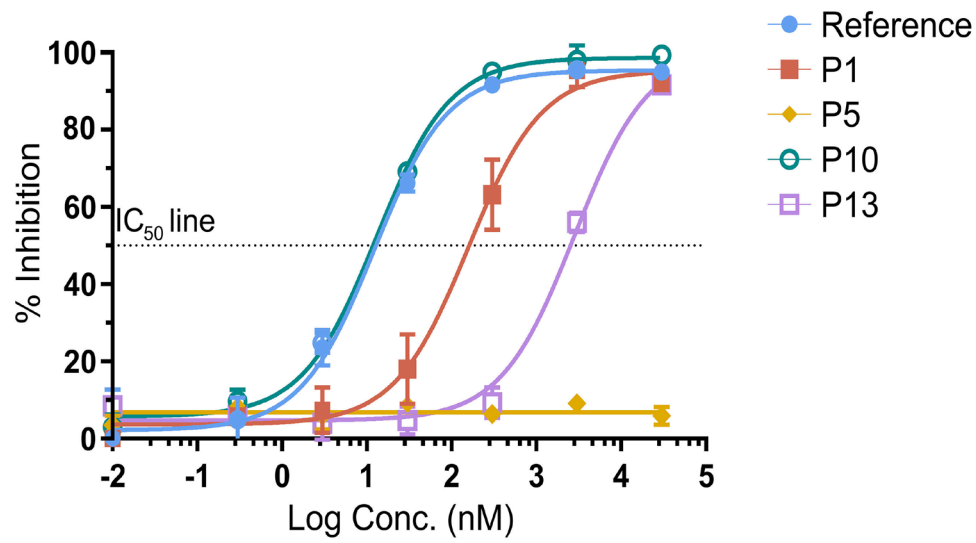
F8	Start	Sequence	DRB1	DPB1
A1	62	SVVYKKTLFVEFTDH	0	3
	73	FTDHLFNIAKPRPPWMG*	1	2
	97	AEVYDTVVITLKNMASHPVS	3	2
	151	GSHTYVWQVLKENGPM	1	0
	222	DEGKSWHSETKNSLMQDRDAASARAWPK	2	0
	240	DAASARAWPKMHTVNG	1	0
a1	379	FDDDNSPSFIQIRSVAKKHPK	7	4
A2	392	SVAKKHPKTWVHYIAAEEEDWDYAPLVLAPDDRSYK	2	4
	474	GEVGDTLLIIFKNQASRPYNIYPHG	7	2
	491	PYNIYPHGITDVRPLYSR	0	2
	576	ESVDQRGNQIMSDKRNVIL*	4	0
	608	ENIQRFLPNPAGVQLEDPEFQ	1	0
a2	740	DSYEDISAYLLSKNNAIEPR	4	2
B	997	AHGPALLTKDNALFKV	2	0
	1131	THGKNSLNSGQGPSPKQLVSLGPEKSVEGQNFLSEKNK*	2	2
	1226	ENVVLPQIHTVTGTKNFMKNL*	1	1
	1668	EITRTTLQSDQEEID*	2	3
A3	1706	SPRSFQKKTRHYFIAAVERLWDYGMSSSPHVL*	6	7
	1724	RLWDYGMSSSPHVLRN*	5	4
	1739	NRAQSGSVPQFKKVVFQEFTD	0	1
	1778	LGPYIRAEVEDNIM	1	0
	1784	AEVEDNIMVTFRNQASRPYSF	7	4
	1820	EPRKNFVKPNETKTYFWKVQ*	1	1
	1900	TIFDETKSWYFTEN	1	0
	1937	FHAINGYIMDTLPGLVMAQDQR	2	0
	1959	IRWYLLSMGSNENIHSIHFSGHVFTVR	0	1
	1984	VRKKEEYKMALYNLYPGVFE	2	11
	1998	YPGVFETVEMLPSK	0	3
C1	2092	VDLLAPMIIHGIKTQGARQK	3	2
	2117	ISQFIIMYSLDGKKWQ	2	1
	2156	HNIFNPPIIARYIRLHPTHYSIR	5	0
C2	2199	MESKAISDAQITASSY	0	1
	2223	SPSKARLHLQGRSNAWRPQ	3	1
	2235	SNAWRPQVNNPKEWLQVDFQKTMK	2	12
	2275	YVKEFLISSSQDGHQWTL	2	2

Factor VIII-derived peptides presented by HLA-DP and HLA-DR molecules were identified using mass spectrometry. The table lists FVIII nested sets identified for HLA-DR and HLA-DP for 11 and 14 donors, respectively. The FVIII nested sets are organized by their sequence localization. The heatmap and the numbers reported in each cell indicate the number of donors in which the FVIII peptides were detected. *Peptides with distinct predicted binding core sequence for HLA-DR as well as distinct predicted binding core sequence for HLA-DP.

investigation of HLA-DP-presented FVIII peptides, with only one study dedicated to this specific aspect.[31]

In this study, we investigated the FVIII peptidome presented on HLA-DP. We employed monocyte-derived dendritic cells (moDC) and incubated these with FVIII. Using a mass-spectrometry based approach, we successfully identified a large repertoire of HLA-DP presented peptides, most of which derived from endogenous proteins (*Online Supplementary Figure S1*). We also explored the repertoire of FVIII-derived peptides that was present on HLA-DP. Interestingly, our findings align with previously published research conducted by Diego *et al.*[31] In agreement with their study, our analysis revealed that a majority of the identified peptides originated from the A3 domain. In addition, 12 out of 14 donors also present peptides derived from the C2 domain (Table 2). We assessed the binding of the HLA-DP-presented peptide repertoire to DPA1*0103/DPB1*0401, which is highly prevalent in the Caucasian population. Evaluation of the binding affinity of naturally presented peptides revealed that the P10 peptide derived from residues 1984-2015 of the A3 domain has the highest affinity for DPA1*0103/DPB1*0401. Seven out of 14 HLA-DP-presented peptides were found to interact with DPA1*0103/DPB1*0401. Interestingly, 7 out of 14 peptides did not bind to DPA1*0103/DPB1*0401. We anticipate that the affinity of these 7 peptides for HLA-DP401 is too low to allow for detection in the binding assay employed in

A



B

Description	SEQUENCE	aa POSITION	FVIII DOMAIN	IC_{50}(nM)
REFERENCE	KKYFAATQFEPLA			11.2
P1MS	SVVYKKTLFVEFTDHL	62-77	A1	151.5
P2MS	PTIQAEVYDTVVITLKNMAS	93-112	A1	2547
P3MS	DDLTDSEMDVVRFDDDNSPSFIQIRSVAKKHPK	367-399	A1	NA
P4MS	GEVGDTLLIIFKNQASRPYNIYPHGI	474-499	A2	4675
P5MS	DSYEDISAYLLSKNNAIEPR	740-759	A2	NA
P6MS	GTKNFMKNLFLLSTRQNVEGSY	1238-1259	B	701.1
P7MS	KKTRHYFIAAVERLWDYGMSSSPHVLRN	1712-1739	A3	NA
P8MS	AEVEDNIMVTFRNQASRPYSF	1784-1804	A3	NA
P9MS	EPRKNFVKPNETKTYFWKVQ	1820-1839	A3	NA
P10MS	VRKKEEYKMALYNLYPGVFETVEMLPSKAGIW	1984-2015	A3	12.01
P11MS	APMIIHGIKTQGARQK	2096-2111	C1	NA
P12MS	MESKAISDAQITASSY	2199-2214	C2	NA
P13MS	VNNPKEWLQVDFQKTMKVTG	2242-2261	C2	3031
P14MS	LTSMYVKEFLISSSQDG	2271-2287	C2	27011

Figure 1. Factor VIII peptides binding affinity to DPA1*0103/DPB1*0401. The dataset of factor (FVIII)-presented peptides was used to create 26 nested sets. Fourteen of 26 nested sets were selected and synthetized (P1 to P14), and their binding affinity to DPA1*0103/DPB1*0401 was tested in a competition assay. The percentage of inhibition (% inhibition) was calculated, and the binding affinity was quantified as IC_{50} values (nM). In the representative graph (A), high affinity binder (P10), low affinity binders (P1, P13), and no binder (P5) were observed. The data for each peptide are summarized in (B). Log Conc: Log-transformed concentration of the non-biotinylated FVIII competitor peptide (0-30 μM). NA: not applicable (i.e., no significant binding observed).

this study. The inclusion of 8 homozygous HLA-DPB*0401 donors allowed us to define a binding motif for this specific allele. Using the Gibbs clustering algorithm, we identified key binding residues for the P1 and P6 position of HLA-DPB1*0401. Peptides presented on this specific molecule preferentially contained a phenylalanine in both P1 and P6 positions (*Online Supplementary Figure S3*). These results are in excellent agreement with findings reported by Castelli and co-workers,[32] which also showed a preference for aromatic amino acids such as phenylalanine at the P1 and the P6 position of the binding pocket of HLA-DP401.

To further investigate the FVIII peptide restriction to HLA-DP, we performed a comparative analysis between the HLA-DR and HLA-DP repertoire on 11 donors. Porcheddu *et al.*[21] investigated specific T-cell response to FVIII peptides in healthy individuals. Sixty-three 20-mer peptides predicted using NetMHCII to bind promiscuously to at least four HLA class II molecules were used in this study. Peptide specific $CD4^+$ T-cell lines were generated and HLA restriction of the resulting $CD4^+$ T-cell clones were evaluated using competition experiments with antibodies specific for HLA-DR (L243), HLA-DP (B7-21), and HLA-DQ (SPVL3). IFN-γ production of one of the clones reactive with peptide P75-94 could be completely blocked with B7-21. In our study, we identify peptides containing a core binding motif present in amino acid sequence 69-86 as being presented by HLA-DP in 2

donors. Interestingly, Diego *et al.* reported that peptides derived from amino acid sequence 69-86 were presented on HLA-DPB1*02:01.[31] These data suggest that peptide sequence P69-86 contains HLA-DP restricted T-cell epitope. Porcheddu *et al.* also reported that proliferation of T-cell clones recognizing peptides P20-39, P178-197, P2082-2103, P2114-2133, P2161-2180, P2226-2245, and P2320-2339 was partially inhibited upon the addition of B7-21.[21] Peptides P2095-2111, P2117-2130, and P2223-2241 were also identified in our study, which is consistent with the presence of HLA-DP restricted T-cell epitopes in these peptides (Table 2).

Remarkably, Porcheddu *et al.* identified $CD4^+$ T-cell clones specific for peptide P1986-2005 which partially overlap with the P10 peptide (P1984-2015) that we identified. The $CD4^+$ T-cell clones recognizing P1986-2005 were fully restricted by HLA-DR.[21] Peptide P2001-2011 was previously identified as being presented on HLA-DQ.[20,31] Peptide P1985-2003 was found to be presented on HLA-DP in agreement with our findings.[20,31] In our study, we identified peptide P1984-2002 on HLA-DR in 2 out of 11 donors. The current data, therefore, suggest that P10 peptide (P1984-2015) may promiscuously bind to HLA-DP, HLA-DQ and HLA-DR potentially via two different core binding motifs (*Online Supplementary Table S1*).

A key limitation of this study is that, although we successfully delineated FVIII-derived peptides using mass spectrometry and validated their binding to HLA-DP4, we did not perform functional assays to confirm which of these peptides act as T-cell epitopes. While some of these peptides have previously been analyzed in T-cell assays, as mentioned earlier, the full set identified in this study has not yet been tested. Therefore, we cannot conclusively determine which peptides are immunogenic or capable of eliciting a T-cell-mediated immune response. Future studies incorporating T-cell functional assays will be essential to validate the immunogenic potential of the entire group of peptides and to clarify their role in triggering immune responses.

Our research provides important insights into the repertoire of FVIII-derived peptides presented on HLA-DP molecules, with a particular focus on HLA-DP4. Unlike previous investigations, which often center on HLA-DR and HLA-DQ, this work represents one of the few in-depth analyses specifically targeting HLA-DP. We successfully validated the repertoire of FVIII peptides naturally presented on HLA-DP and demonstrated which peptides exhibit strong binding affinities to HLA-DP4. This focused approach offers a deeper understanding of peptide presentation by HLA-DP and also contributes to our knowledge of presentation of FVIII-derived peptides on MHC class II.

Moreover, the identification of promiscuous FVIII peptides, capable of being presented by multiple HLA class II molecules, including HLA-DP, HLA-DQ, and HLA-DR, holds significant promise in the context of epitope-based therapies. Our findings suggest that incorporating HLA-DP4-presented peptides into such therapies could be highly advantageous, particularly for developing novel peptide-based tolerization strategies aimed at targeting a broad population of Caucasian individuals with hemophilia A.

Disclosures

No conflicts of interest to disclose.

Contributions

MM, BEH and JV designed the study. MM performed the experiments. MM performed the data analysis. BEH established the setup of the competition assay. BW and VP helped in the peptide selection, in silico analyses, and biological assays. FvA assisted with the mass spectrometry experiments. PK helped in the purification of pdFVIII. MM and JV wrote the manuscript. KF, SLD, MvdB and BM provided valuable input to the study. All authors critically reviewed the manuscript.

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Data-sharing statement

Data included in this study will be made available by the authors on request. Mass spectrometry data generated in this study will be submitted to the PRIDE-consortium repository.

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