haematologica
Journal of the Ferrata Storti Foundation

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

by Mariarosaria Miranda, Bjarke Endel Hansen, Batoul Wehbi, Valeria Porcheddu, Floris P.J. van Alphen, Paul Kaijen, Karin Fijnvandraat, Sebastien Lacroix-Desmazes, Maartje van den Biggelaar, Bernard Maillere, and Jan Voorberg.
Collaborative Groups: EDUC8 Consortium (Flora Peyvandi, Christoph Koenigs, Johannes Oldenburg, Yvette van Kooyk, Bernard Maillère, Sebastien Lacroix-Desmazes, Jan Voorberg).

Received: July 3, 2024.
Accepted: December 2, 2024.

Citation: Mariarosaria Miranda, Bjarke Endel Hansen, Batoul Wehbi, Valeria Porcheddu, Floris P.J. van Alphen, Paul Kaijen, Karin Fijnvandraat, Sebastien Lacroix-Desmazes, Maartje van den Biggelaar, Bernard Maillere, and Jan Voorberg.
Collaborative Groups: EDUC8 Consortium (Flora Peyvandi, Christoph Koenigs, Johannes Oldenburg, Yvette van Kooyk, Bernard Maillère, Sebastien Lacroix-Desmazes, Jan Voorberg).
FVIII peptides presented on HLA-DP and identification of an A3 domain peptide binding with high affinity to the commonly expressed HLA-DP4.
Haematologica. 2024 Dec 12. doi: 10.3324/haematol.2024.286204 [Epub ahead of print]

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

Mariarosaria Miranda[1], Bjarke Endel Hansen[2], Batoul Wehbi[3], Valeria Porcheddu[3], Floris P.J. van Alphen[1], Paul Kaijen[1], Karin Fijnvandraat[1,4], Sebastien Lacroix-Desmazes[5], Maartje van den Biggelaar[1], Bernard Maillere[3] and Jan Voorberg[1,6], on behalf of the EDUC8 consortium.

Collaborative Groups: EDUC8 Consortium (Flora Peyvandi, Christoph Koenigs, Johannes Oldenburg, Yvette van Kooyk, Bernard Maillère, Sebastien Lacroix-Desmazes, Jan Voorberg)

1. Department of Molecular Hematology, Sanquin Research and Landsteiner Laboratory, Amsterdam, The Netherlands
2. Immudex, Copenhagen, Denmark
3. Université Paris-Saclay, CEA, INRAE, Departement Médicaments et Technologies pour la Santé, SIMoS, Gif-sur-Yvette, France
4. Amsterdam University Medical Center location University of Amsterdam, Department of Pediatric Hematology, Amsterdam, The Netherlands
5. Institut National de la Santé et de la Recherche Médicale, Centre de Recherche des Cordeliers, CNRS, Sorbonne Université, Université Paris Cité, Paris, France
6. Department of Experimental Vascular Medicine, Amsterdam University Medical Center, Amsterdam, The Netherlands

Author 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 peptides selection, in silico analyses and biological assays. FvA assisted with the MS 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.

Running head

FVIII peptide repertoire presented on HLA-DP

Corresponding author

Jan Voorberg, Department of Molecular Hematology, Sanquin Research and Landsteiner Laboratory, Amsterdam, The Netherland; e-mail: j.voorberg@sanquin.nl

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.

Acknowledgements

We thank Liselotte Brix for helpful discussions and facilitating peptide binding studies performed at Immudex.

Funding information

This project has received funding from the European Union's Horizon 2020 research and innovation program under the Marie Slodowska-Curie grant agreement no. 859974 (EDUC8). Additional funding was obtained from the Ministry of Health grant PPOC-23-22.

Disclosures

The author(s) declare no financial relationships with commercial entities during the 36 months prior to publication. No patents related to the work described in this manuscript have been submitted.

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 (APCs), B- and T-helper lymphocytes. APCs 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 monocytes-derived dendritic cells (moDCs) from HLA-typed healthy donors. Using this method, we identified 176 to 1352 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. 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 (DCs) play a crucial role as antigen-presenting cells (APCs) in the immune response to FVIII. DCs take up FVIII antigens through various endocytic processes and present FVIII-derived peptides on MHC class II molecules. Mature DCs 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 cell 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 moDCs FVIII-loaded mature monocyte-derived dendritic (moDCs) cells 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 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 *(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 2h 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 1h at 2-8°C. After washing of 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 IC50 values calculated for the FVIII peptides, based on their competition with the reference peptide. Data were reported as IC50 values. Peptides with IC50 values below 1 µM were considered to bind with high affinity.

Data 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 FVIII-derived peptides presented on HLA-DP

To explore the FVIII peptides presented on HLA-DP, we employed monocytes-derived DCs (moDCs) from 14 HLA-typed healthy donors. Immature moDCs 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 moDCs 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 1000 HLA-DP-associated

peptides from seven 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 (**Supplementary Figure S1a**), biological process (**Supplementary Figure S1b**), and molecular function (**Supplementary Figure S1c**). Enrichment analysis revealed terms with the highest score with emphasis on the adjusted p-value columns as a prominent scoring metric (**Supplementary Figure 1a**). Our findings show that cell adhesion molecules and other protein binding complexes are commonly presented on HLA-DP (**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] (**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 DCs were incubated in the presence and absence of FVIII (donors E, F, G, H, I, J, K, L, M and N). Immature DCs 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 1352 distinct peptides (**Table 1**). As expected FVIII derived peptides were presented only on FVIII-pulsed moDCs. 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** displays 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 2467 (**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 16000 HLA-DR presented peptides and over 9000 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 molecules present a broader and more diverse array of peptides compared to HLA-DP.

The repertoire of FVIII 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, as shown in the **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 were 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 bind to either HLA-DR and HLA-DP (**Supplementary table 1**)."

Identification of peptide binding motifs to HLA-DP401

Eight of the donors included in this study were homozygous for HLA-DPB1*0401. This allowed for assessing 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 (**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 donor 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) (**Supplementary Figure S3, left panel**). 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 acids 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 observed in positions 2 and 7 (**Supplementary Figure S3, right panel**). 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 (personal communication Prof. M. Nielsen).

Binding affinity of FVIII 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 IC50 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 IC50 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 (**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 DCs 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 DCs, 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 candidate 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 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 (moDCs) 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 (**Supplementary Figure 1**). 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 to low to allow for detection in the binding assay employed in 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 position (**Supplementary Figure 3**). These result 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 P6 position of the binding pocket of HLA-DP401.

To further investigate the FVIII peptides 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 line 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-□ 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 (**Supplementary Table 1**).

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 toour knowledge on 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.

References

1. Mannucci PM. Hemophilia treatment innovation: 50 years of progress and more to come. J Thromb Haemost. 2023;21(3):403-412.

2. Pratt KP, Arruda VR, Lacroix-Desmazes S. Inhibitors-Recent insights. Haemophilia. 2021;27(S3):28-36.

3. Lacroix-Desmazes S, Voorberg J, Lillicrap D, Scott DW, Pratt KP. Tolerating Factor VIII: Recent Progress. Front Immunol. 2019;10:2991.

4. Childers KC, Peters SC, Spiegel PC. Structural insights into blood coagulation factor VIII: Procoagulant complexes, membrane binding, and antibody inhibition. J Thromb Haemost. 2022;20(9):1957-1970.

5. Mancuso ME, Mahlangu JN, Pipe SW. The changing treatment landscape in haemophilia: from standard half-life clotting factor concentrates to gene editing. Lancet. 2021;397(10274):630-640.

6. Mahlangu J, Iorio A, Kenet G. Emicizumab state-of-the-art update. Haemophilia. 2022;28(Suppl 4):103-110.

7. Meeks SL, Zimowski KL. Haemophilia in the era of novel therapies: Where do inhibitors feature in the new landscape? Haemophilia. 2024;30(Suppl 3):95-102.

8. Pratt KP. Inhibitory antibodies in hemophilia A. Curr Opin Hematol. 2012;19(5):399-305.

9. Kaczmarek R, Pineros AR, Patterson PE, et al. Factor VIII Trafficking to CD4+ T cells Shapes its Immunogenicity and Requires Several Types of Antigen Presenting Cells. Blood. 2023;142(3):290-305.

10. Matino D, Tieu P, Chan A. Molecular mechanisms of inhibitor development in hemophilia. Mediterr J Hematol Infect Dis. 2020;12(1):e2020001.

11. Prezotti ANL, Frade-Guanaes JO, Yamaguti-Hayakawa GG, Ozelo MC. Immunogenicity of Current and New Therapies for Hemophilia A. Pharmaceuticals (Basel). 2022; 15(8):911.

12. Ettinger RA, Liberman JA, Gunasekera D, et al. FVIII proteins with a modified immunodominant T-cell epitope exhibit reduced immunogenicity and normal FVIII activity. Blood Adv. 2018;2(4):309-322.

13. Winterling K, Martin WD, De Groot AS, Daufenbach J, Kistner S, Schüttrumpf J. Development of a novel fully functional coagulation factor VIII with reduced immunogenicity utilizing an in silico prediction and deimmunization approach. J Thromb Haemost. 2021;19(9):2161-2170.

14. Pletinckx K, Nicolson KS, Streeter HB, et al. Antigen-specific immunotherapy with apitopes suppresses generation of FVIII inhibitor antibodies in HLA-transgenic mice. Blood Adv. 2022;6(7):2069-2080.

15. Maldonado RA, LaMothe RA, Ferrari JD, et al. Polymeric synthetic nanoparticles for the induction of antigen-specific immunological tolerance. Proc Natl Acad Sci U S A. 2015;112(2):E156-165.

16. Kelly A, Trowsdale J. Genetics of antigen processing and presentation. Immunogenetics. 2019;71(3):161-170.

17. Jankowski W, Park Y, McGill J, et al. Peptides identified on monocyte-derived dendritic cells: a marker for clinical immunogenicity to FVIII products. Blood Adv. 2019;3(9):1429-1440.

18. van Haren SD, Herczenik E, ten Brinke A, Mertens K, Voorberg J, Meijer AB. HLA-DR-presented Peptide Repertoires Derived From Human Monocyte-derived Dendritic Cells Pulsed With Blood Coagulation Factor VIII. Mol Cell Proteomics. 2011;10(6):M110.002246.

19. van Haren SD, Wroblewska A, Herczenik E, et al. Limited Promiscuity of HLA-DRB1 Presented Peptides Derived of Blood Coagulation Factor VIII. PLoS One. 2013;8(11):e80239.

20. Peyron I, Hartholt RB, Pedró-Cos L, et al. Comparative profiling of HLA-DR and HLA-DQ associated factor VIII peptides presented by monocyte-derived dendritic cells. Haematologica. 2018;103(1):172-178.

21. Porcheddu V, Lhomme G, Giraudet R, Correia E, Maillère B. The self-reactive FVIII T cell repertoire in healthy individuals relies on a short set of epitopes and public clonotypes. Front Immunol. 2024;15:1345195.

22. Reding MT, Wu H, Krampf M, et al. CD4+ T cell response to factor VIII in hemophilia A, acquired hemophilia, and healthy subjects. Thromb Haemost. 1999;82(2):509-515.

23. Ettinger RA, James EA, Kwok WW, Thompson AR, Pratt KP. Lineages of human T-cell clones, including T helper 17/T helper 1 cells, isolated at different stages of anti-factor VIII immune responses. Blood. 2009;114(7):1423-1428.

24. Ettinger RA, James EA, Kwok WW, Thompson AR, Pratt KP. HLA-DR-restricted T-cell responses to factor VIII epitopes in a mild haemophilia A family with missense substitution A2201P. Haemophilia. 2010;16(102):44-55.

25. James EA, Kwok WW, Ettinger RA, Thompson AR, Pratt KP. T-cell responses over time in a mild hemophilia A inhibitor subject: epitope identification and transient immunogenicity of the corresponding self-peptide. J Thromb Haemost. 2007;5(12):2399-2407.

26. Ettinger RA, Liberman JA, Gunasekera D, et al. FVIII proteins with a modified immunodominant T-cell epitope exhibit reduced immunogenicity and normal FVIII activity. Blood Adv. 2018;2(4):309-322.

27. Ettinger RA, Paz P, James EA, et al. T cells from hemophilia A subjects recognize the same HLA-restricted FVIII epitope with a narrow TCR repertoire. Blood. 2016;128(16):2043-2054.

28. Jacquemin M, Vantomme V, Buhot C, et al. CD4+ T-cell clones specific for wild-type factor VIII: a molecular mechanism responsible for a higher incidence of inhibitor formation in mild/moderate hemophilia A. Blood. 2003;101(4):1351-1358.

29. Meunier S, Menier C, Marcon E, Lacroix-Desmazes S, Maillère B. CD4 T cells specific for factor VIII are present at high frequency in healthy donors and comprise naïve and memory cells. Blood Adv 2017;1(21):1842-1847.

30. Menier C, Meunier S, Porcheddu V, et al. Frequency of natural regulatory T cells specific for factor VIII in the peripheral blood of healthy donors. Eur J Immunol. 2024;54(4):e2350506.

31. Diego VP, Luu BW, Hofmann M, et al. Quantitative HLA-class-II/factor VIII (FVIII) peptidomic variation in dendritic cells correlates with the immunogenic potential of therapeutic FVIII proteins in hemophilia A. J Thromb Haemost. 2020;18(1):201-216.

32. Castelli FA, Buhot C, Sanson A, et al. HLA-DP4, the Most Frequent HLA II Molecule, Defines a New Supertype of Peptide-Binding Specificity. J Immunol. 2002;169(12):6928-6934.

33. Shaw S, Ziegler A, DeMars R. Specificity of monoclonal antibodies directed against human and murine class II histocompatibility antigens as analyzed by binding to HLA-deletion mutant cell lines. Hum Immunol. 1985;12(4):191-211.

34. Trombetta ES, Mellman I. Cell biology of antigen processing in vitro and in vivo. Annu Rev Immunol. 2005;23:975-1028.

35. Falk K, Rotzschke O, Stevanovic S, Jung G, Rammensee H-G. Pool sequencing of natural HLA-DR, DQ, and DP ligands reveals detailed peptide motifs, constraints of processing, and general rules. Immunogenetics. 1994;39(4):230-242.

36. Witmer C, Young G. Factor VIII inhibitors in hemophilia A: rationale and latest evidence. Ther Adv Hematol. 2013;4(1):59-72.

37. Anderson RP, Jabri B. Vaccine against autoimmune disease: antigen-specific immunotherapy. Curr Opin Immunol. 2013;25(3):410-417.

38. Pearce SHS, Dayan C, Wraith DC, et al. Antigen-Specific Immunotherapy with Thyrotropin Receptor Peptides in Graves' Hyperthyroidism: A Phase I Study. Thyroid. 2019;29(7):1003-1011.

39. Prakken BJ, Samodal R, Le TD, et al. Epitope-specific immunotherapy induces immune deviation of proinflammatory T cells in rheumatoid arthritis. Proc Natl Acad Sci U S A. 2004;101(12):4228-4233.

40. Koffeman EC, Genovese M, Amox D, et al. Epitope-specific immunotherapy of rheumatoid arthritis: Clinical responsiveness occurs with immune deviation and relies on the expression of a cluster of molecules associated with T cell tolerance in a double-blind, placebo-controlled, pilot phase II trial. Arthritis Rheum. 2009;60(11):3207-3216.

41. Chataway J, Martin K, Barrell K, Sharrack B, Stolt P, Wraith DC. Effects of ATX-MS-1467 immunotherapy over 16 weeks in relapsing multiple sclerosis. Neurology. 2018;90(11):e955-e962.

42. Goel G, King T, Daveson AJ, et al. Epitope-specific immunotherapy targeting CD4-positive T cells in coeliac disease: two randomised, double-blind, placebo-controlled phase 1 studies. Lancet Gastroenterol Hepatol. 2017;2(7):479-493.

43. Zhang A-H, Rossi RJ, Yoon J, Wang H, Scott DW. Tolerogenic nanoparticles to induce immunologic tolerance: Prevention and reversal of FVIII inhibitor formation. Cell Immunol. 2016;301:74-81.

Table 1

	HLA DRB1*	HLA DPB1*	HLA-DRB1*			HLA-DPB1*			HLA-DRB1*			HLA-DPB1*		
			no FVIII			no FVIII			FVIII			FVIII		
Donors	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

Table 1. The repertoire of naturally presented peptides on HLA-DP. 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 FVIII and FVIII-pulsed moDCs. The tables include genotype information, the number of proteins, unique peptides, and FVIII peptides 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.

Table 2a

			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														

Table 2b

			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														
		NNPKEWLQVDFQKTMKVT														
		NPKEWLQVDFQKT														
		NPKEWLQVDFQKTM														
		NPKEWLQVDFQKTMK														
		NPKEWLQVDFQKTMKV														
		NPKEWLQVDFQKTMKVT														
		NPKEWLQVDFQKTMKVTG														
	2271	LTSMYVKEFLISSSQ														
		LTSMYVKEFLISSSQDG														
		SMYVKEFLISSSQ														
		YVKEFLISSSQDGHQW														

Table 2. Complete list of FVIII peptides on HLA-DP. FVIII derived peptides on HLA-DP were identified using mass spectrometry based approach. The complete list of FVIII peptides from 14 donors sorted on their sequence localization is displayed. **Table 2a and 2b** show FVIII peptides belonging to the heavy (A1-*a1*-A2-*a2*-B) and light chains (*a3*-A3-C1-C2), respectively. Each column represents the results for an individual donor. Peptides identified for each donor are depicted in green.

Table 3

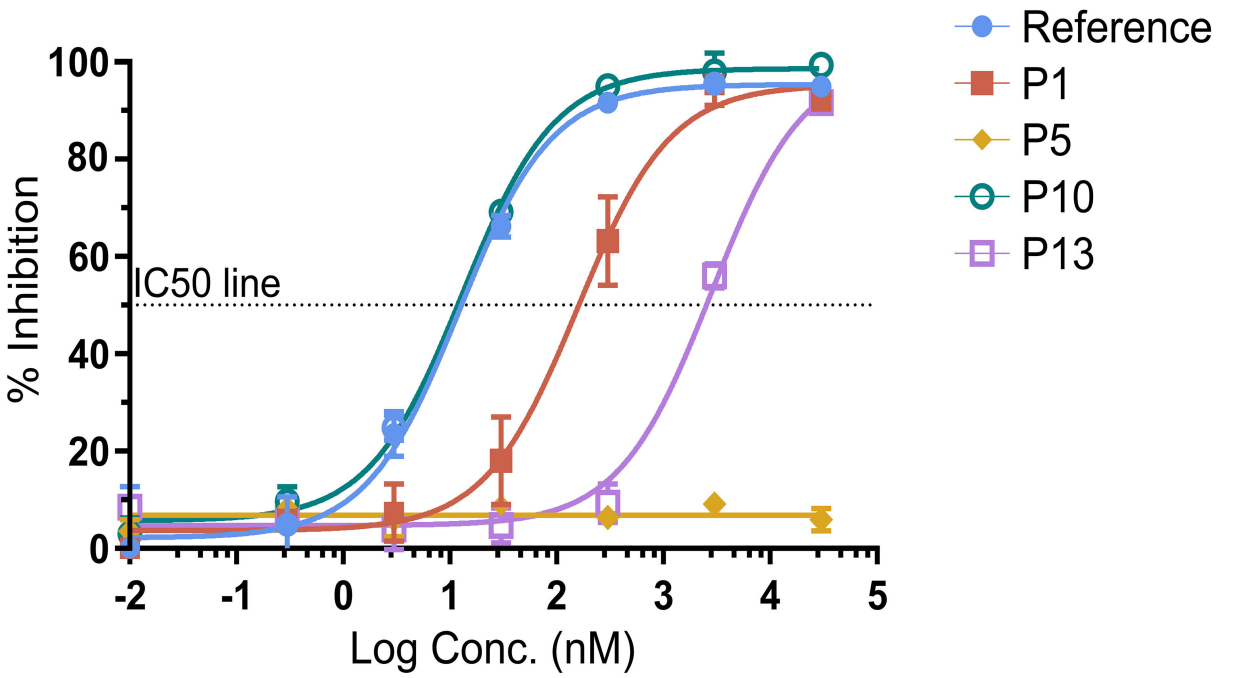
F8	Start	Sequence	DRB1	DPB1
A1	62	SVVYKKTLFVEFTDH	0	3
	73	FTDHLFNIAKPRPPWMG*	1	2
	97	AEVYDTVVITLKNMASHPVS	3	2
	151	GSHTYVWQVLKENGPM	1	0
	222	DEGKSWHSETKNSLMQDRDAASARAWPK	2	0
	240	DAASARAWPKMHTVNG	1	0
a1	379	FDDDNSPSFIQIRSVAKKHPK	7	4
A2	392	SVAKKHPKTWVHYIAAEEEDWDYAPLVLAPDDRSYK	2	4
	474	GEVGDTLLIIFKNQASRPYNIYPHG	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

Table 3. Comparison of FVIII peptides on HLA-DP and HLA-DR. FVIII-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. Asterisks indicate peptides with distinct predicted binding core sequence for HLA-DR as well as distinct predicted binding core sequence for HLA-DP.

Figure legend

Figure 1. FVIII peptides binding affinity to DPA1*0103/DPB1*0401. The dataset of 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 IC50 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 panel b. NA (not applicable); no significant binding observed.

A



B

Description	SEQUENCE	aa POSITION	FVIII DOMAIN	IC50 (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

Supplementary Methods

Reagents Plasma derived FVIII (Aafact) was purified as described previously[1]; FVIII derived peptides (Proteogenix, TAG Copenhagen A/S); CD14 microbeads, and manual MACS Magnetic Separators for cell separation from Miltenyi; Cellgro DC serum-free medium, rh-IL-4, and rh-GM-CSF from CellGenix; LPS from Sigma-Aldrich; bulk anti-Human HLA-DP antibody (B7/21) from Ichorbio; InVivoMAb anti-human/monkey HLA-DR (L243) from (BioXCell); Human Serum Albumin (HSA) (200 g/l) from Sanquin; and CNBr Sepharose 4B (GE Healthcare) were used for this study. Purified monoclonal antibodies (mAb) B7/21 and L243 were coupled to CNBr Sepharose 4B at a final concentration of 2 mg/ml. The anti-human HLA-DR, -DQ and -DP (IVA12), were purified from IVA12 hybridoma (ATCC) supernatant on a protein A Sepharose (GE Healthcare)[2].

Preparation of monocyte derived dendritic cells and factor VIII Endocytosis Peripheral blood mononuclear cells (PBMC) were isolated from freshly drawn blood from HLA-typed healthy volunteers by separation over a Ficoll-Paque™ PLUS gradient (GE Healthcare). Monocytes were isolated from the PBMC fraction by positive selection using CD14 microbeads and a magnetic cell separator. Monocytes were cultured at a concentration of $1x10^6$ cells/ml in a 6-well plate in Cellgro medium supplemented with GM-CSF (1000 IU/ml) and IL-4 (800 IU/ml) for 5 days. After 5 days of culture, the immature moDCs were washed and replated in Cellgro medium supplemented with GM-CSF and IL-4 at a concentration of $2.5x10^6$ cells/ml in a final volume of 2 ml. Immature dendritic cells were incubated in the presence or absence of 100 nM FVIII for 5h. Subsequently, the immature moDCs were maturated by adding LPS to a final concentration of 1 µg/ml and human serum albumin to a final concentration of 1% to the culture medium overnight.

Mass spectrometry Samples were desalted using C18 STAGE tips which were prepared in house. STAGE tips were equilibrated with 100% acetonitrile and washed with 1% formic acid. Samples were loaded on STAGE tips and washed once with 1% formic acid and once with 1% formic acid supplemented with 5% acetonitrile. Peptides were eluted from STAGE tips with 60 µL 1% formic acid/30% acetonitrile and concentrated to a final volume of 5 µL using vacuum centrifugation. Peptides were separated using an in-house prepared column filled with 1.9 µm C18 particles (New Objective type FS360-75-8-N-5-C20) at a flow rate of 300 nL/min, with a step-wise gradient from 0 to 72 % (v/v) acetonitrile containing 0.1 % formic acid. Column eluate was sprayed directly into the Orbitrap Fusion™ Lumos™ Tribrid™ Mass Spectrometer (ThermoFisher) using a nanoelectrospray source with a spray voltage of 2.15 kV. Higher-energy collisional dissociation was performed in top-speed mode with 3 second cycles (400-1500 m/z, resolving power 120,000). Tandem mass spectrometry was performed by isolation with the quadrupole with isolation window 0.7 and rapid scan mass spectrometry analysis in the ion trap. The MS^2 ion count target was set to 3×10^4 and the max injection time was 20 ms. Only those precursors with charge state 2–7 were sampled for MS^2. The dynamic exclusion duration was set to 20 s with a 10 ppm tolerance around the selected precursor and its isotopes. All data were acquired with Xcalibur software. The mass spectrometer was calibrated on a regular basis to ensure a high mass accuracy.

Data analysis The functional enrichment analysis was performed using g:Profiler (version e111_eg58_p18_30541362) with g:SCS multiple testing correction method applying significance threshold of 0.05[34]. GibbsCluster – 2.0 was used for unsupervised alignment and clustering of peptide sequences. NetMHCII–4.3 was used to predict binding of peptides to MHC class II.

References

1. Bril WS, van Helden PMW, Hausl C, et al. Tolerance to factor VIII in a transgenic mouse expressing human factor VIII cDNA carrying an Arg593 to Cys substitution. Thromb Haemost 2006;95(2):341–347.

2. Shaw S, Ziegler A, DeMars R. Specificity of monoclonal antibodies directed against human and murine class II histocompatibility antigens as analyzed by binding to HLA-deletion mutant cell lines. Hum Immunol 1985;12(4):191–211.

Figures Legend

Supplementary Figure 1. Gene Ontology Enrichment analysis of proteins identified for HLA-DP molecule. Endogenous and processed proteins presented on HLA-DP were annotated according to their cellular compartment (**A**), biological process (**B**), and molecular function (**C**) using a gene ontology enrichment analysis. The tables highlights the terms with the lowest adjusted p-value. These terms are ranked based on their significance, with only the most significantly enriched terms included in the table. The color scale indicates the magnitude of the adjusted p-value.

Supplementary Figure 2. FVIII peptides binding affinity to DPA1*0103/DPB1*0401. The dataset of FVIII naturally presented peptides was used to create 26 nested sets. One representative peptide from 14 of 26 nested sets was 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 IC50 values (nM). The graphs illustrate the binding trend for each peptide.

Supplementary Figure 3. Binding motifs to HLA-DP. To identify the binding motifs to HLA-DP, the Gibbs clustering algorithm was applied. As input, we used the list of naturally presented peptides from donor A, G, H, I, J, K, L, M and N, who are homozygous for HLA-DPB1*04:01. Cluster 2 had the highest Kullback-Leibler Distance(KLD) (**left panel**). SeqLogo (**right panel**) displays consensus sequences, where Group 1 shows weak conservation (bit score up to 0.4) and Group 2 shows stronger conservation (bit score up to 0.6). In Group 2, phenylalanine (F) residues were prominently present at positions 2 and 7.

Supplementary Table 1. Comparison of FVIII peptides on HLA-DP and HLA-DR. FVIII-derived peptides presented by HLA-DP and HLA-DR molecules were identified using mass spectrometry. The table lists FVIII binding cores to the MHC class II for fourteen donors, which were predicted using NetMHCIIpan4.3. The FVIII core peptides were sorted by their sequence localization and shown separately for HLA-DP and HLA-DR.

Supplementary Figure 1

A

Cellular compartment

ID	Term name	p_{adj}
1	vesicle	4.464×10^{-197}
2	extracellular organelle	3.178×10^{-190}
3	extracellular membrane-bounded organelle	3.178×10^{-190}
4	extracellular vesicle	1.855×10^{-189}
5	extracellular exosome	2.691×10^{-189}
6	extracellular space	4.216×10^{-156}
7	extracellular region	1.878×10^{-133}
8	intracellular vesicle	3.907×10^{-119}
9	cytoplasmic vesicle	4.142×10^{-118}
10	cytoplasm	1.453×10^{-107}
11	secretory granule	2.804×10^{-93}
12	endomembrane system	4.446×10^{-88}
13	secretory vesicle	2.936×10^{-86}
14	cell-substrate junction	1.692×10^{-69}
15	vesicle lumen	2.260×10^{-69}
16	focal adhesion	3.583×10^{-69}
17	secretory granule lumen	4.195×10^{-69}
18	cytoplasmic vesicle lumen	1.618×10^{-68}
19	membrane	1.028×10^{-58}
20	lytic vacuole	1.096×10^{-54}
21	lysosome	1.096×10^{-54}
22	vacuole	5.433×10^{-53}
23	anchoring junction	1.100×10^{-51}
24	ficolin-1-rich granule	2.332×10^{-51}
25	cytosol	2.853×10^{-51}
26	bounding membrane of organelle	4.946×10^{-44}
27	cell junction	7.623×10^{-44}
28	ficolin-1-rich granule lumen	1.145×10^{-39}
29	organelle membrane	3.900×10^{-38}
30	cytosolic ribosome	5.896×10^{-38}
31	vesicle membrane	7.714×10^{-38}
32	cytoplasmic vesicle membrane	1.600×10^{-37}
33	membrane-bounded organelle	1.723×10^{-37}
34	pigment granule	3.401×10^{-37}
35	melanosome	3.401×10^{-37}
36	endocytic vesicle	2.344×10^{-35}
37	endosome	9.326×10^{-35}
38	cell surface	7.023×10^{-34}
39	azurophil granule	9.147×10^{-34}
40	primary lysosome	9.147×10^{-34}
41	organelle	1.718×10^{-32}
42	vacuolar lumen	2.485×10^{-31}
43	cell periphery	1.305×10^{-30}
44	secretory granule membrane	3.040×10^{-29}
45	tertiary granule	4.828×10^{-29}
46	endoplasmic reticulum	1.709×10^{-27}
47	vacuolar membrane	2.686×10^{-26}
48	lytic vacuole membrane	9.255×10^{-26}
49	lysosomal membrane	9.255×10^{-26}
50	intracellular membrane-bounded organelle	1.130×10^{-25}

B

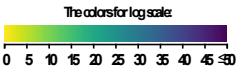
Biological process

ID	Term name	p_{adj}
1	organonitrogen compound metabolic process	3.044×10^{-46}
2	protein metabolic process	2.579×10^{-41}
3	transport	2.055×10^{-38}
4	cytoplasmic translation	3.673×10^{-37}
5	localization	6.703×10^{-37}
6	response to stimulus	1.073×10^{-35}
7	response to stress	1.830×10^{-35}
8	establishment of localization	1.397×10^{-33}
9	vesicle-mediated transport	1.466×10^{-32}
10	biological process in interspecies interation	6.986×10^{-32}
11	response to external stimulus	8.501×10^{-31}
12	response to organic substance	4.463×10^{-30}
13	catabolic process	5.617×10^{-30}
14	immune system process	6.692×10^{-30}
15	cellular response to chemical stimulus	9.057×10^{-30}
16	endocytosis	1.873×10^{-29}
17	response to chemical	3.509×10^{-29}
18	amide metabolic process	2.989×10^{-28}
19	regulation of response to stimulus	1.222×10^{-27}
20	defense response	1.611×10^{-27}
21	peptide metabolic process	5.206×10^{-27}
22	regulation of biological quality	1.017×10^{-26}
23	cellular localization	2.102×10^{-26}
24	import into cell	2.305×10^{-26}
25	regulation of protein metabolic process	7.369×10^{-26}
26	positive regulation of biological process	9.219×10^{-26}
27	cellular catabolic process	4.663×10^{-25}
28	regulation of multicellular organismal process	2.201×10^{-24}
29	macromolecule localization	4.949×10^{-24}
30	cellular response to stimulus	2.949×10^{-23}
31	regulation of localization	2.994×10^{-23}
32	cell migration	3.915×10^{-23}
33	immune response	4.404×10^{-23}
34	cell adhesion	5.980×10^{-23}
35	response to biotic stimulus	1.080×10^{-22}
36	response to other organism	1.908×10^{-22}
37	positive regulation of response to stimulus	2.394×10^{-22}
38	response to external biotic stimulus	2.450×10^{-22}
39	positive regulation of cellular process	4.601×10^{-22}
40	cell motility	5.834×10^{-22}
41	organic substance catabolic process	7.200×10^{-22}
42	cell activation	1.238×10^{-21}
43	organic substance transport	2.498×10^{-21}
44	regulation of cell adhesion	3.175×10^{-21}
45	regulation of signaling	4.996×10^{-21}
46	regulation of immune system process	1.591×10^{-20}
47	regulation of signal transduction	2.293×10^{-20}
48	nitrogen compound transport	2.539×10^{-20}
49	positive regulation of protein metabolic process	2.812×10^{-20}
50	regulation of cell communication	5.502×10^{-20}

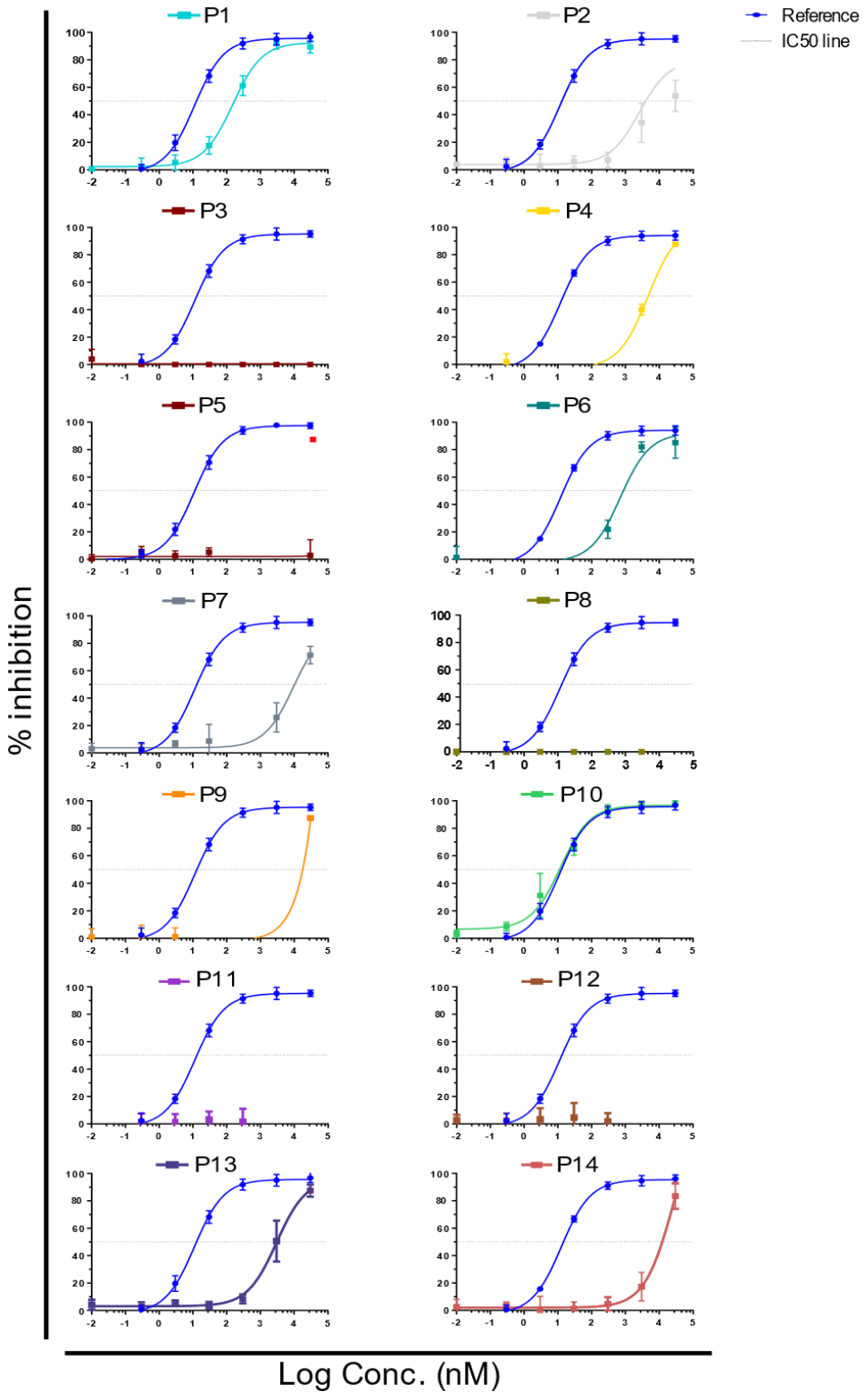
C

Molecular function

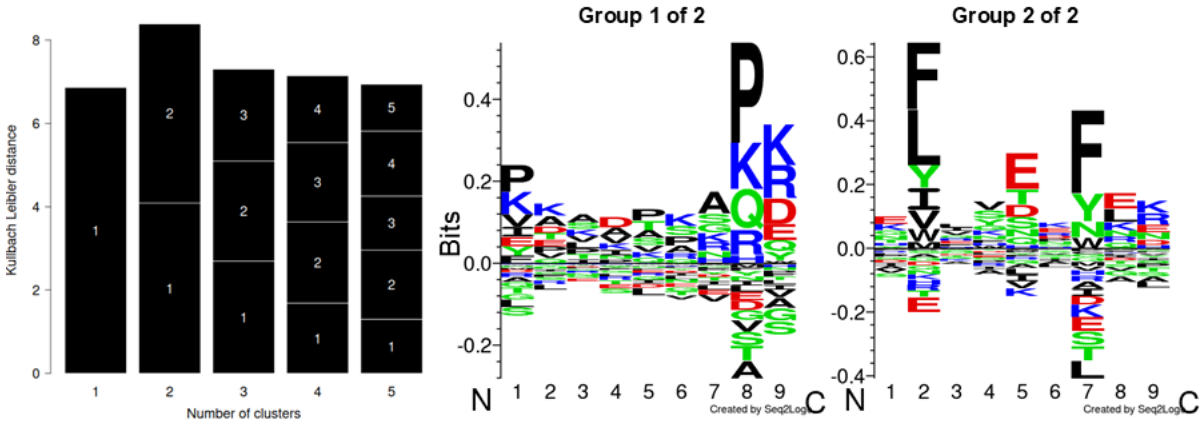
ID	Term name	p_{adj}
1	protein binding	1.877×10^{-50}
2	cell adhesion molecule binding	4.925×10^{-32}
3	protein-containing complex binding	9.867×10^{-27}
4	cadherin binding	6.466×10^{-24}
5	enzyme binding	1.175×10^{-22}
6	identical protein binding	2.587×10^{-16}
7	signaling receptor binding	8.604×10^{-16}
8	hydrolase activity	1.424×10^{-12}
9	carbohydrate derivative binding	2.812×10^{-11}
10	actin binding	5.261×10^{-11}
11	structural molecule activity	1.043×10^{-10}
12	cytoskeletal protein binding	1.124×10^{-10}
13	actin filament binding	5.092×10^{-10}
14	amide binding	5.516×10^{-10}
15	catalytic activity	5.573×10^{-10}
16	MHC class II protein complex binding	7.270×10^{-10}
17	anion binding	8.167×10^{-10}
18	peptide binding	2.102×10^{-9}
19	binding	3.898×10^{-9}
20	small molecule binding	7.240×10^{-9}
21	protein dimerization activity	9.942×10^{-9}
22	carbohydrate binding	1.212×10^{-8}
23	MHC protein complex binding	2.546×10^{-8}
24	protein domain specific binding	1.199×10^{-7}
25	lipid binding	1.566×10^{-7}
26	heterocyclic compound binding	1.649×10^{-7}
27	integrin binding	3.557×10^{-7}
28	calcium ion binding	5.049×10^{-7}
29	exogenous protein binding	5.548×10^{-7}
30	nucleoside phosphate binding	8.561×10^{-7}
31	protein homodimerization activity	9.475×10^{-7}
32	antioxidant activity	9.480×10^{-7}
33	ion binding	1.141×10^{-6}
34	nucleotide binding	1.355×10^{-6}
35	molecular function inhibitor activity	1.676×10^{-6}
36	structural constituent of cytoskeleton	1.846×10^{-6}
37	virus receptor activity	2.476×10^{-6}
38	enzyme inhibitor activity	2.485×10^{-6}
39	exopeptidase activity	3.227×10^{-6}
40	mRNA binding	5.753×10^{-6}
41	lipoprotein particle receptor binding	5.775×10^{-6}
42	amyloid-beta binding	8.596×10^{-6}
43	ubiquitin-like protein ligase binding	1.048×10^{-5}
44	ubiquitin protein ligase binding	1.205×10^{-5}
45	kinase binding	1.638×10^{-5}
46	GTP binding	1.760×10^{-5}
47	growth factor binding	2.224×10^{-5}
48	purine ribonucleoside triphosphate binding	2.345×10^{-5}
49	protein phosphatase binding	2.570×10^{-5}
50	protease binding	2.570×10^{-5}



Supplementary Figure 2



Supplementary Figure 3



Supplementary Table 1

		DONOR A		DONOR B		DONOR C		DONOR D		DONOR E	DONOR F	DONOR G	DONOR H		DONOR I		DONOR J		DONOR K		DONOR L		DONOR M		DONOR N	
F8	Start	DRB1	DPB1	DRB1	DPB1	DRB1	DPB1	DRB1	DPB1	DPB1	DPB1	DPB1	DRB1	DPB1	DRB1	DPB1	DRB1	DPB1	DRB1	DPB1	DRB1	DPB1	DRB1	DPB1	DRB1	DPB1
A1	65 78 104 155 235 247		YKKTLFVEF							FTDHLFNIA VITLKNMAS		YKKTLFVEF FTDHLFNIA	VITLKNMAS LMQDRDAAS			YKKTLFVEF	FNIAKPRPP YVWQVLKEN WPKMHTVNG		VITLKNMAS LMQDRDAAS						VITLKNMAS	VITLKNMAS
a1	387 401	FIQIRSVAK		FIQIRSVAK		FIQIRSVAK					FIQIRSVAK	FIQIRSVAK	FIQIRSVAK WVHYIAAEE WDYAPLVLA	FIQIRSVAK WVHYIAAEE WDYAPLVLA		WVHYIAAEE WDYAPLVLA	YAPLVLAPD	WVHYIAAEE	FIQIRSVAK	FIQIRSVAK WVHYIAAEE	FIQIRSVAK		FIQIRSVAK			
A2	481 494 583 610	LIIFKNQAS NQIMSDKRN		NQIMSDKRN								LIIFKNQAS	LIIFKNQAS	LIIFKNQAS	LIIFKNQAS MSDKRNVI IQRFLPNPA		LIIFKNQAS		LIIFKNQAS		LIIFKNQAS IMSDKRNVI		LIIFKNQAS	IYPHGITDV ITDVRPLYS		IYPHGITDV ITDVRPLYS
a2	748					YLLSKNNAI	YLLSKNNAI	YLLSKNNAI	YLLSKNNAI				YLLSKNNAI						YLLSKNNAI							
B	1001 1148 1233							ALLTKDNAL				FMKNLFLLS	LVSLGPEKS		LTKDNALFK		LVSLGPEKS IHTVTGTKN	LVSLGPEKS		VEGQNFLSE					
A3	1669 1715 1728 1749 1781 1826 1902 1943 1972 1990 2002	MVTFRNQAS	YKMALYNLY		YKMALYNLY	YFIAAVERL GMSSSPHVL FDETKSWYF			RHYFIAAV YKMALYNLY	YKMALYNLY	RHYFIAAV	MVTFRNQAS ETKTYFWKV YKMALYNLY	YFIAAVERL YGMSSSPHV MVTFRNQAS	ITRTTLQSD RHYFIAAVE GMSSSPHVL MVTFRNQAS YKMALYNLY FETVEMLPS	MSSSPHVLR MVTFRNQAS VKPNETKTY	ITRTTLQSD RHYFIAAVE MSSSPHVLR YKMALYNLY	YFIAAVERL YGMSSSPHV YIRAEVEDN MVTFRNQAS YKMALYNLY	RTTLQSDQE FQKKTRHYF RHYFIAAVE MSSSPHVLR MVTFRNQAS YKMALYNLY FETVEMLPS	ITRTTLQSD FQKKTRHYF YFIAAVERL YGMSSSPHV MVTFRNQAS MALYNLYPG	RHYFIAAVE MSSSPHVLR FKKVVFQEF MVTFRNQAS YKMALYNLY FETVEMLPS	VERLWDYGM MVTFRNQAS	RHYFIAAVE	YIMDTLPGL	IHSIHFSGH YKMALYNLY	FQKKTRHYF YIMDTLPGL	YKMALYNLY
C1	2100 2120 2168									IHGIKTQGA			IHGIKTQGA FIIMYSLDG IRLHPTHYS		IRLHPTHYS		IRLHPTHYS		IHGIKTQGA FIIMYSLDG IRLHPTHYS	IHGIKTQGA FIIMYSLDG					IHGIKTQGA IRLHPTHYS	
C2	2204 2229 2241 2279		WLQVDFQKT		WLQVDFQKT		WLQVDFQKT		ISDAQITAS WLQVDFQKT	WLQVDFQKT FLISSSQDG	WLQVDFQKT	WLQVDFQKT	LHLQGRSNA FLISSSQDG	WLQVDFQKT	LHLQGRSNA	WLQVDFQKT	LHLQGRSNA	LHLQGRSNA WLQVDFQKT	WLQVDFQKT FLISSSQDG ISSSQDGHQ	WLQVDFQKT FLISSSQDG		WLQVDFQKT	QVNNPKEWL			