Comparative profiling of HLA-DR and HLA-DQ associated factor VIII peptides presented by monocyte-derived dendritic cells

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ABSTRACT

The development of anti-factor VIII antibodies is a major complication of the treatment of patients with hemophilia A. Generation of high affinity anti-factor VIII antibodies is dependent on help provided by CD4+ T cells that recognize factor VIII-derived peptides presented on class II major histocompatibility complex on the surface of antigen-presenting cells. In order to identify the immune-dominant epitopes that can be presented to CD4+ T cells, we previously developed a mass spectrometry-based method to identify factor VIII-derived peptides that are presented on human leukocyte antigen (HLA)-DR. In the present work, we compared the repertoire of FVIII-derived peptide presented on HLA-DR and HLA-DQ. Monocyte-derived dendritic cells from nine HLA-typed healthy donors were pulsed with recombinant factor VIII. HLA-DR and HLA-DQ molecules were purified using monoclonal antibodies. Our data show that HLA-DQ and HLA-DR present a similar repertoire of factor VIII-derived peptides. However, the number of peptides associated with HLA-DQ was lower than that with HLA-DR. We also identified a peptide, within the acidic a3 domains of factor VIII, which is presented with higher frequency on HLA-DQ. Interestingly, this peptide was found to have a higher predicted affinity for HLA-DQ than for HLA-DR. Taken together, our data suggest that HLA-DQ participates in the presentation of factor VIII peptides, thereby contributing to the development of inhibitory antibodies in a proportion of patients with severe hemophilia A.

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Introduction

Hemophilia A is an X-linked coagulation disorder characterized by decreased levels of functional factor VIII (FVIII) in circulation. In the most severe form of hemophilia A (FVIII residual activity <1 IU/dL), the absence of functional endogenous FVIII leads to spontaneous bleeding episodes and life-threatening hemorrhages.[1] To compensate for the lack of endogenous FVIII, therapeutic FVIII is administered intravenously to the patients either on-demand or under prophylaxis. While the current treatment protocols are successful in most patients and lead to a symptom-free, near-normal life expectancy[2], about 30% of patients develop an immune response to the administered FVIII.[3-6] The humoral response against therapeutic FVIII results in the generation of anti-FVIII antibodies that inhibit the pro-coagulant activity of FVIII (FVIII inhibitors). This is a serious complication of the treatment of

patients with hemophilia A.[7] FVIII inhibitors are predominantly of the IgG1 and IgG4 isotypes,[8] suggesting that the anti-FVIII immune response is dependent on help provided by CD4+ T cells. Activation of FVIII-specific CD4+ T cells requires the internalization of FVIII by professional antigen-presenting cells, such as dendritic cells, macrophages or B cells. After intracellular processing, FVIII-derived peptides are presented at the cell surface in association with major histocompatibility class II (MHCII) molecules. The first signal leading to the activation of CD4+ T cells is provided by the interaction of the T-cell receptor with peptide-bound MHCII on the surface of antigen-presenting cells. Together with the expression of co-stimulatory molecules, the presentation of FVIII peptides by antigen-presenting cells primes and activates FVIII-specific CD4+ T cells. Subsequently, the FVIII-specific CD4+ T cells recognize peptide/MHCII complexes on the B-cell surface resulting in the activation of FVIII-specific B cells that differentiate into anti-FVIII IgG producing plasma cells or FVIII-specific memory B cells.[9] Several genetic and non-genetic risk factors have been associated with the incidence of inhibitor development.[10–17] Among them, the HLA haplotype of patients has been linked to the presence of FVIII inhibitors.[13–16] Located on the short arm of chromosome 6, the class II HLA gene complex contains three loci, DP, DQ and DR. Each of these loci encodes at least one alpha chain (DPA, DQA and DRA, respectively) and a variable number of beta chain polypeptides (DPB, DRB and DQB, respectively). As of December 2016, 4,230 HLA class II alleles had been assigned, half of which are attributed to variations in DRB.[18] Less than 10% of these alleles are commonly identified in unrelated individuals as described in the Common and Well Documented (CWD) catalogue assembled by the American Society for Histocompatibility and Immunogenetics (ASHI).[19] A larger allele variation can be identified in Europe.[20] Hence, the 2017 European Federation for Immunogenetics (EFI) CWD HLA catalogue reported a total of 1,048 CWD alleles. Sanchez-Mazas and co-workers identified 130 DRB1, 20 DQA1 and 86 DQB1 alleles.[20] Since HLA class II molecules arise from the non-covalent association of non-identical alpha and beta chains, up to 130 different HLA-DR and 1720 HLA-DQ haplotypes can be found in the general European population.[20]

As mentioned earlier, various studies have investigated the association of HLA haplotypes with the presence of inhibitors. In several studies the haplotypes HLA-DRB1*15 and DQB1*0602 were significantly associated with inhibitor development.[13,16] In general it appeared that HLA profile was not a major determinant for inhibitor development.[13] This may potentially be due to the wide repertoire of FVIII peptides that can be presented on HLA class II. Alternatively, promiscuous binding of immunodominant peptides to multiple HLA molecules may explain the absence of a strong link between HLA profile and inhibitor development. Identification of the immunodominant T-cell epitopes of FVIII which are presented on HLA molecules is still a major challenge.[21] Using a mass spectrometry-based approach, our group previously identified the immunodominant peptides derived from FVIII presented on HLA-DR.[22,23] The aim of the present work was to address the repertoire of FVIII-derived peptides presented on HLA-DQ and to compare it to that presented on HLA-DR. Using monoclonal antibodies specific for HLA-DR or HLA-DQ, we characterized for the first time the repertoire of FVIII-derived peptides presented on HLA-DQ.

Methods

Purification of peptide/MHC complexes

Blood was obtained after approval from the Sanquin Ethical Advisory Board, in accordance with the Declaration of Helsinki. FVIII-loaded mature monocyte-derived dendritic 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 complete protease inhibitor) for 30 min at 4°C. Lysates were then centrifuged at 20,000xg for 15 min at 4°C and incubated with 300 μL of Sepharose beads coupled with either anti-HLA-DR (L243) or anti HLA-DQ (SPV-L3) monoclonal antibodies. 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 using 10% acetic acid for 10 min at room temperature. Eluates were collected and heated for 15 min at 70°C to dissociate the peptide/MHCII complexes.

Mass spectrometry

Samples were desalted using C18 STAGE tips prepared in-house. STAGE tips were equilibrated with 100% acetonitrile and subsequently 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 supplemented with 30% acetonitrile and concentrated to a final volume of 5 μL using vacuum centrifugation. Eluted peptides were separated using columns filled with 1.9 μm C18 particles (New Objective type FS360-75-8-N-5-C20, Inc., Woburn, MA, USA) at a flow rate of 300 nL/min, with a step-wise gradient from 0 to 72 % (v/v) acetonitrile in 0.1 M acetic acid. Column eluate was sprayed directly into the Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific Inc., Bremen, Germany) using a nano-electrospray 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). The mass spectrometer was calibrated on a regular basis as recommended by the manufacturer in order to ensure a high mass accuracy.

Data analysis

Peptides were identified using Proteome Discoverer 1.4 (Thermo Scientific, Bremen, Germany). Raw Xcalibur data files were screened against the UniprotKB non-redundant protein 25.H_sapiens.fase database 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. The NetMHCIIpan 3.1 server[24] was used to determine the binding core sequence of overlapping FVIII peptides identified by mass spectrometry as outlined in the *Online Supplementary Information*. The binding core sequence with highest affinity was used as the representative peptide for each group of identified peptides irrespectively of the associated HLA allele. Differences in peptide numbers were statistically assessed with a two-sided non-parametric Mann-Whitney U-test using Graphpad Prism 7.0. The heat map was generated with Perseus 1.5.6.0.[25] The absolute numbers of uniquely identified peptides for each protein were clustered with the following settings: distance - Euclidean, linkage - average, number of clusters - 300, maximal number of iterations - 10, number of restarts - 1.

Results

Comparative analysis of peptide presentation by HLA-DR and HLA-DQ

In order to explore the repertoire of FVIII-derived peptides presented on HLA-DR and HLA-DQ, we generated monocyte-derived dendritic cells from nine healthy donors. The dendritic cells were pulsed with 100 nM of recombinant full-length FVIII and maturation was induced using lipopolysaccharide to stabilize the expression of HLA-peptide complexes at the cell surface. Subsequently, cells were lysed and HLA-DR or HLA-DQ molecules were purified using Sepharose beads conjugated with the monoclonal antibodies L243 (HLA-DR) or SPV-L3 (HLA-DQ). Samples were then analyzed using mass spectrometry. As depicted in Figure 1A, the total number of peptides found in the case of HLA-DQ was consistently lower than that in the case of HLA-DR (733.7 ± 216.3 *versus* 1724 ± 491.4, mean ± SD). Similarly, the number of FVIII-derived peptides eluted from HLA-DQ was lower than that eluted from HLA-DR (7.8 ± 7.6 *versus* 36.7 ± 17.2, mean ± SD, Figure 1B). We then investigated the relative expression of HLA-DR and HLA-DQ. As shown in Figure 1C, we found a 4-fold lower expression of HLA-DQ compared to HLA-DR on monocyte-derived dendritic cells, suggesting that the lower abundance of the peptides eluted from HLA-DQ compared to HLA-DR is due to the lower expression of HLA-DQ.

In order to determine the selectivity of peptide presentation by HLA-DR and HLA-DQ, the proteins identified by their unique peptides were clustered based on their absolute peptide count for all donors. The top 40 clustered proteins identified in the case of HLA-DR and HLA-DQ were visualized in a heat map (Figure 2). Consistent with our previous observation, the overall number of peptides found was lower in the case of HLA-DQ compared to HLA-DR. Peptides derived from positively charged histones were preferentially associated with HLA-DQ. In contrast, HLA-derived peptides were primarily found to be presented on HLA-DR. Based on these findings, HLA-DR and HLA-DQ appear to present distinct but overlapping peptide repertoires. In addition, FVIII was the sixth hit in the case of HLA-DR, but was found only at position 35 in the heat map generated for HLA-DQ, suggesting that FVIII-derived peptides preferentially associate with HLA-DR.

The repertoire of factor VIII-derived peptides presented on HLA-DQ overlaps with that on HLA-DR

In order to compare the profiles of FVIII-derived peptides presented on HLA-DR and HLA-DQ, the binding cores of the unique FVIII-derived peptides were determined using NetMHCIIpan 3.1.[24] For the nine donors, core peptides were predicted with respect to their HLA haplotypes. FVIII-derived peptides sharing the same predicted core were grouped. An overview of the predicted cores is shown in Figure 3 for the nine donors tested (A to I) for HLA-DR and HLA-DQ. The complete set of FVIII-derived peptides is provided in *Online Supplementary Figure S1*. To compare the repertoire of FVIII-derived peptides presented on HLA-DR and HLA-DQ, the predicted cores of FVIII-derived peptides identified in the nine donors were grouped. As shown in Figure 4A, most of the peptides identified to be presented on HLA-DQ were also identified on HLA-DR, suggesting that the repertoire of FVIII-derived peptides presented on HLA-DQ overlaps with that presented on HLA-DR. Of note, with the exception of one donor, no B domain-derived peptides were found to be presented on HLA-DQ.

A peptide derived from the acidic a3 domains is efficiently presented on HLA-DQ

To investigate the reasons underlying the reduced number of FVIII peptides found to be presented on HLA-DQ, we compared the predicted affinities of the FVIII-derived peptides identified in both the HLA-DR and HLA-DQ

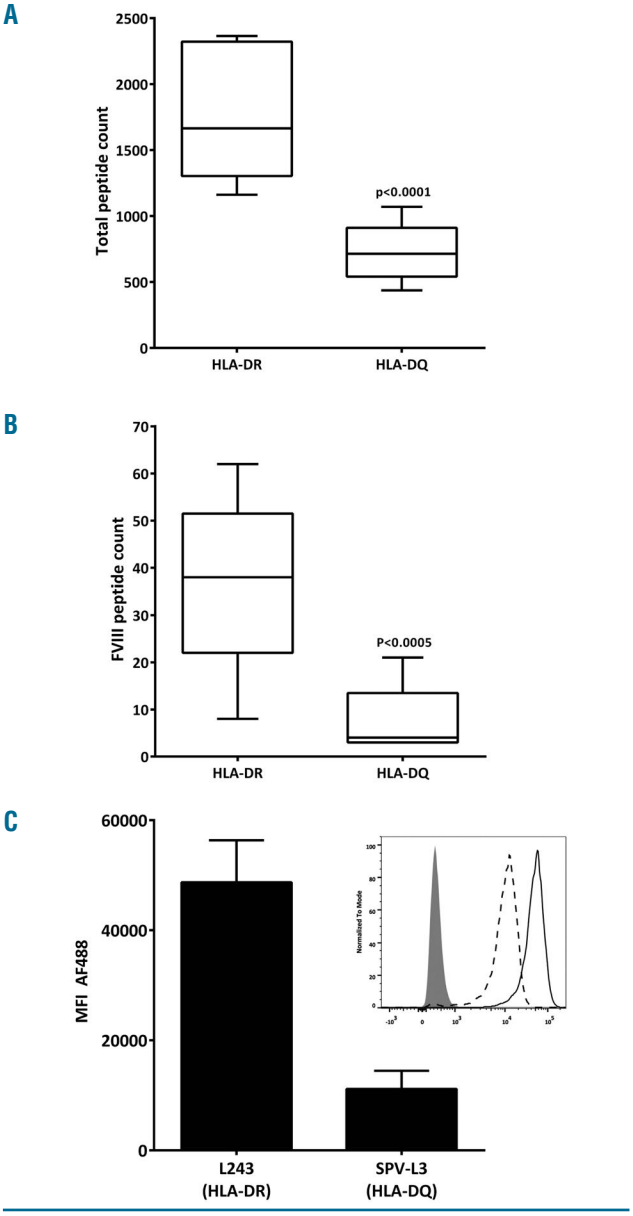

Figure 1. Comparative profiling of peptide presentation by HLA-DR and HLA-DQ. Five million monocyte-derived dendritic cells were pulsed with 100 nM FVIII and matured for 24 h. Peptides associated with HLA-DR and HLA-DQ were identified using mass spectrometry. (A) Panel A shows the total number of peptides identified after elution from either HLA-DR or HLA-DQ. (B) Panel B depicts the FVIII-specific peptides found on either HLA-DR or HLA-DQ. Statistical differences were determined using the non-parametric Mann-Whitney U-test. (C) Two hundred thousand immature monocyte-derived dendritic cells were incubated with 5 μg/mL of monoclonal antibody L243 (HLA-DR) or SPV-L3 (HLA-DQ). The relative binding of L243 and SPV-L3 was determined by flow cytometry using Alexa Fluor 488-conjugated anti-mouse IgG2a secondary antibody. Inset: representative histogram of the flow cytometry analysis of HLA-DR and HLA-DQ expression.

pull-downs with respect to the HLA type of the corresponding donors. As depicted in Figure 4B, the majority of the FVIII-derived peptides identified have a higher predicted affinity for HLA-DR than for HLA-DQ. Together with the lower expression of HLA-DQ, the relatively low affinity of these FVIII-derived peptides for HLA-DQ could explain their under-representation in the HLA-DQ pull-downs. Interestingly, a peptide, derived from the acidic a3 domain displayed a higher affinity for HLA-DQ than for HLA-DR (Figure 4B). This peptide was identified with a similar frequency in HLA-DQ and HLA-DR eluates (Figure 4A). These observations suggest that this a3-derived peptide is preferentially presented on HLA-DQ.

Discussion

In the present work we describe for the first time the repertoire of FVIII-derived peptides presented on HLA-DQ by monocyte-derived dendritic cells. In parallel, we also determined the repertoire of FVIII-derived peptides presented on HLA-DR. Overall, fewer peptides were presented on HLA-DQ than on HLA-DR. This observation was correlated with a 4-fold lower surface expression of HLA-DQ compared to HLA-DR. Our results are consistent with previous publications reporting lower RNA levels of the DQA1 chain compared to DRA1[26] and a lower surface expression of HLA-DQ compared to HLA-DR.[27] In addition, while the dendritic cells used for HLA-DR and HLA-DQ immunoprecipitation were incubated with equal amounts of FVIII, comparative analysis of the total FVIII-derived peptides presented on HLA-DR and HLA-DQ revealed a preferential presentation of FVIII-derived peptides on HLA-DR. This suggests that following FVIII processing, HLA-DR and HLA-DQ molecules within the endolysosomal compartment compete for the binding of FVIII-derived peptides. Consistent with previous work describing the repertoire of naturally presented peptides by mature monocyte-derived dendritic cells, we identified a large collection of peptides derived from proteins from intracellular compartments which were presented on HLA-DR and HLA-DQ.[23,28–30] Peptides derived from endogenous proteins are expected to compete with FVIII-derived peptides for binding to MHCII. Indeed, our analysis of the peptides presented on HLA-DQ revealed the presentation of peptides derived from several other endogenous proteins prior to FVIII, further limiting the presentation of FVIII-derived peptides on HLA-DQ. While the overall presentation of FVIII-derived peptides seemed to be more limited on HLA-DQ compared to HLA-DR, we identified a set of peptides within the acidic a3 domain which was equally presented on both HLA-DR and HLA-DQ. Interestingly, the predicted affinities of this peptide identified an MHCII binding core with higher affinities for HLA-DQ than for HLA-DR alleles. This suggests that the lower expression of HLA-DQ can be compensated by a higher affinity of these peptides for HLA-DQ.

The predicted affinity of the FVIII peptides identified in this study for HLA-DR and HLA-DQ varies considerably (see Figure 4B). A subset of HLA-DR-presented peptides is predicted to bind with high affinity to HLA-DR. In contrast, the predicted affinity for FVIII peptides presented on HLA-

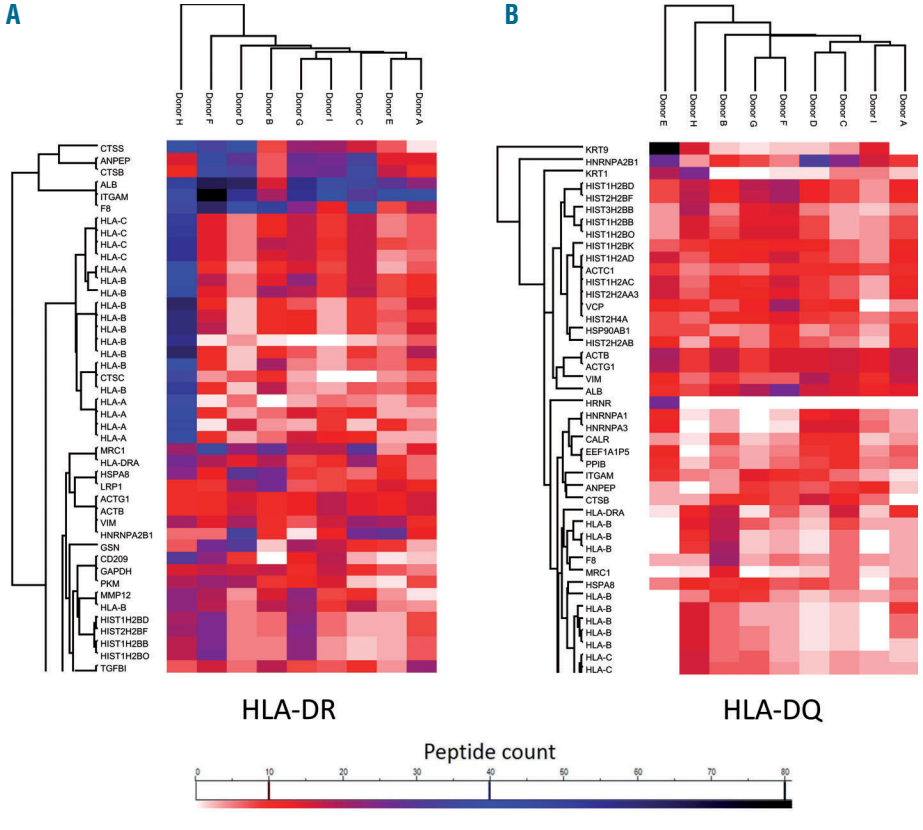

Figure 2. Cluster analysis of proteins identified for HLA-DR and HLA-DQ. The absolute counts of unique peptide from all donors were grouped using hierarchical clustering for HLA-DR (left panel) and HLA-DQ (right panel). The top 40 proteins are displayed (black ≥ 80 peptides, blue = 40 peptides, red ≤ 10 peptides). Gene names (referred to as UniProt) are used to represent the proteins.

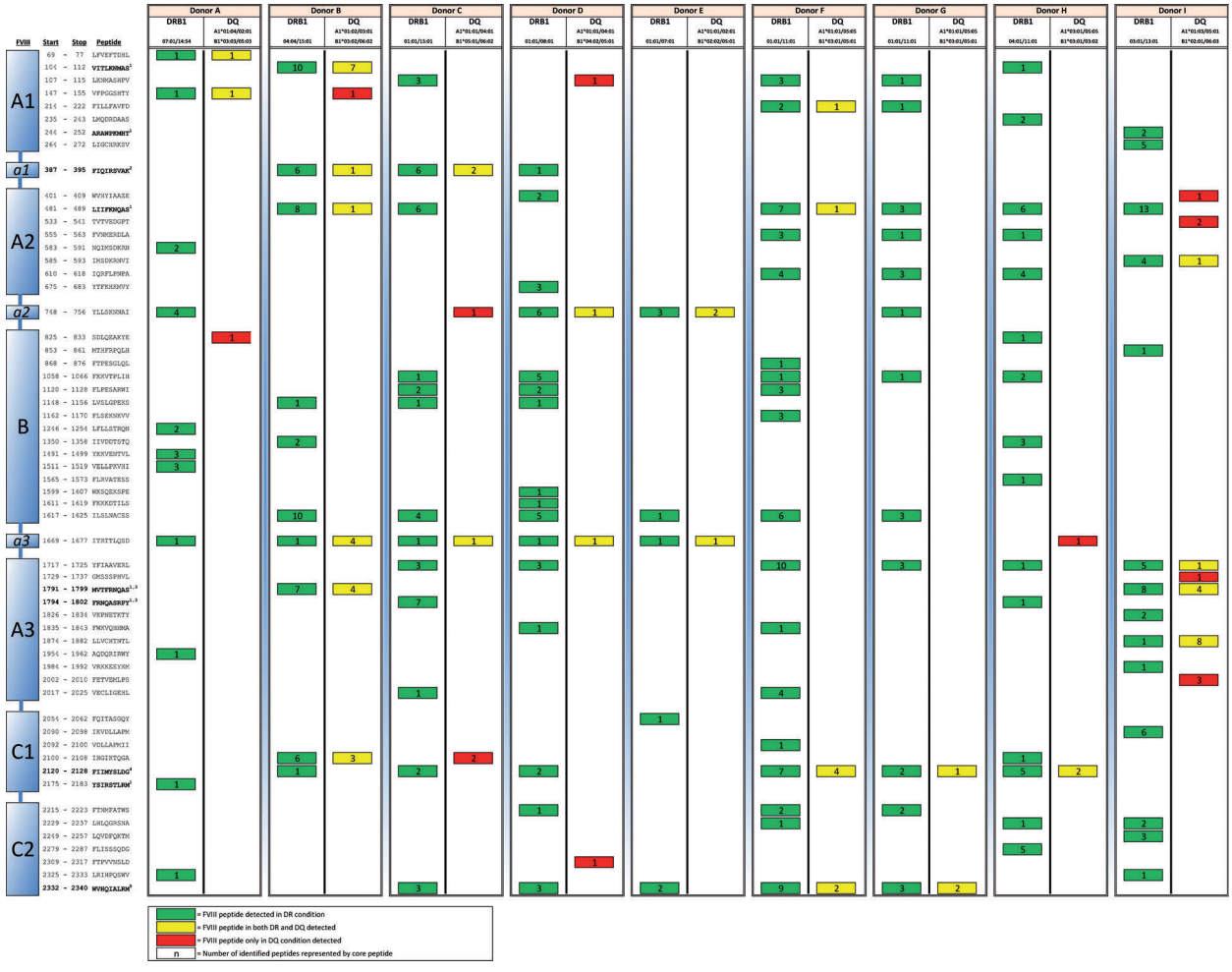

Figure 3. Overview of factor VIII peptides presented by HLA-DR and HLA-DQ. FVIII-derived peptides presented on HLA-DR and HLA-DQ were identified using mass spectrometry. Core-peptide sequences and affinities were determined using NetMHCIIpan 3.1 for the different HLA-DR and HLA-DQ combinations. The core peptide with highest affinity was selected as the representative sequence for each group of overlapping peptides. In the case of overlapping discrepancies in the core-peptide sequence between HLA-DR and HLA-DQ, the core peptide determined for HLA-DR was used for the graphic representation. Core peptides were sorted based on their sequence localization. Each column represents the results for an individual donor. Green: peptide identified in the HLA-DR condition. Yellow: peptide identified in the HLA-DR and HLA-DQ condition. Red: peptide identified only in the HLA-DQ condition. Numbers within boxes indicate the total number of peptides represented by the specific core peptide. Core sequences indicated in bold correspond to previously documented T-cell epitopes: [1]Steinitz *et al.*, Blood 2012; [2]Hu *et al.*, JTH, 2004; [3]Reding *et al.*, JTH, 2004; [4]Jones *et al.*, JTH, 2005; [5]Reding *et al.*, JTH, 2003.

DQ appears to be quite low with an overall predicted affinity of >100 nM (Figure 4B). The significance of these observations is currently unknown. The strength of association between the T-cell receptor and the peptide-MHC complex was repeatedly found to dictate the activation and polarization of T cells.[31,32] Very few reports have addressed the contribution of the peptide affinity for the HLA molecule to the strength of association between the T-cell receptor and peptide-MHC complex. Evidence has been obtained, in a model of influenza infection, in support of antigen signal strength during the priming of CD4$^+$ T cells playing a crucial role for the cells' effector function.[33] In that study, it was argued that peptide-MHC stability has a significant impact on the functional properties of the effector CD4$^+$ T cells.[33] Similarly, the range of predicted affinities of the FVIII peptides identified in this study could guide the development of functionally diverse CD4$^+$ T cells.

The predicted lower affinity of HLA-DQ-presented peptides would need to be confirmed by *in vitro* peptide-binding assays. The affinity of a small number of FVIII peptides for different MHCII molecules has been experimentally determined. A C1 domain-derived peptide Ile2163-Thr2180 bound with high affinity (IC_{50} below 20 nM) to several DRB1 molecules.[34] In contrast, a much lower affinity (IC_{50} ranging from 0.5 to 1.0 μM) was measured for binding of an A2 domain-derived peptide (Gln611-Leu622) to DRB1*11.[35] The range of predicted affinities for the FVIII peptides identified in this study is consistent with the experimentally determined values. It is, therefore, likely that the set of FVIII peptides identified in this study is representative of the repertoire of FVIII peptides that is presented on antigen-presenting cells of patients with hemophilia A (and most likely by default also on antigen-presenting cells of healthy individuals). Our current knowledge about the peptide repertoire recognized by FVIII-specific CD4$^+$ T cells in hemophilia A patients with inhibitors is still limited. Nevertheless, the repertoire of experimentally determined CD4$^+$ T-cell epitopes over-

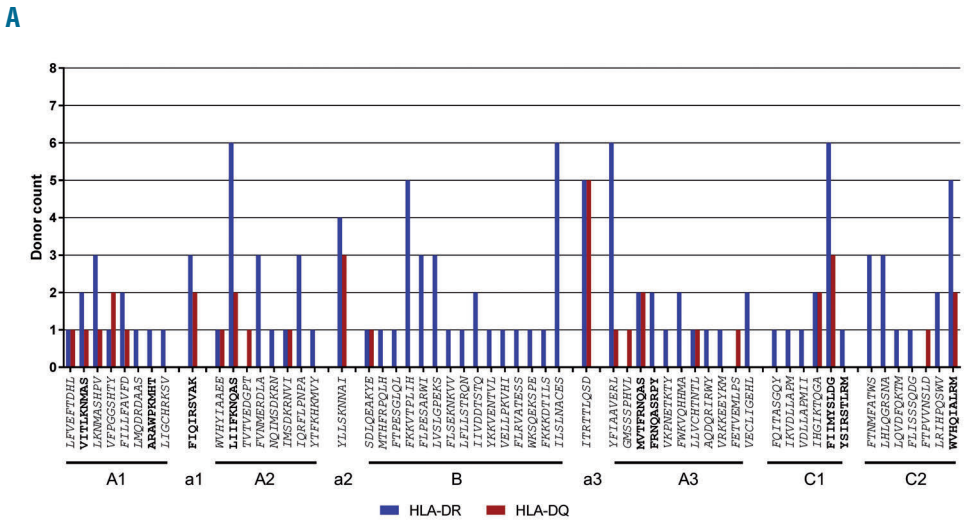

Figure 4. Comparison of the factor VIII-derived peptide repertoire presented on HLA-DR and HLA-DQ. (A) The data obtained from the nine donors were grouped based on core-peptide sequence and their frequency (out of 9). Blue and red bars represent the frequency with which the FVIII core peptide was found in the HLA-DR and HLA-DQ conditions, respectively. Core sequences indicated in bold correspond to previously documented T-cell epitopes. (B) The affinity of the peptides found in both the HLA-DR and HLA-DQ conditions was determined using NetMHCIIpan 3.1 and plotted as paired peptides. Data points corresponding to the acidic a3 peptides are depicted in red.

laps significantly with the repertoire of naturally presented FVIII peptides as identified in this and previous studies.[22,23] This is exemplified by the peptides with core sequences of VITLKNMAS, ARAWPKNHT, LIIFKNQAS, FRNQASRPY and YSIRSTLRM, which were previously identified in a humanized E17 HLA-DRB1*15:01 mouse model.[36] While these peptides were found associated with HLA-DRB1*15:01, we also detected these peptides on non-DRB1*15:01 HLA molecules. Conversely, several peptides identified as $CD4^+$ T-cell epitopes by Steinitz and co-workers could not be detected by our experimental approach. This may be due to potential limitations in our current experimental protocols.

In the present study, a higher-energy collision-induced dissociation strategy was employed for peptide fragmentation. Recently, Mommen *et al.* employed multiple peptide fragmentation technologies (electron-transfer dissociation, higher-energy collision-induced dissociation and combined electron-transfer/higher-energy collision dissociation) to successfully expand the HLA ligandome.[29,37] In the present work, C18 STAGE tips were used to process HLA-DR and HLA-DQ eluates. The use of multi STAGE-Tip consisting of a hydrophobic C18 disk combined with a strong cation exchange disk could potentially increase the diversity of identified peptides by allowing for binding for peptides with different chemical properties.[38]

While HLA haplotypes are commonly investigated as a genetic risk factor in various human diseases, very few studies have linked the expression levels of HLA gene products with the susceptibility to or outcome of autoimmune diseases. This was exemplified by Cavalli *et al.*, who did not associate the susceptibility to auto-immune vitiligo development with a specific HLA haplotype, but with three single nucleotide polymorphisms located in a region that regulates the expression of the HLA genes.[39] Several studies have investigated the relation between HLA haplotypes (especially DRB1) and the presence of inhibitor in hemophilia A patients, yielding variable results.[13–16] Whether the other three HLA-DRB encoding-genes (DRB3, DRB4 and DRB5) as well as HLA expression levels participate in the pathogenesis of inhibitor formation in patients with hemophilia A remains to be established.

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