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ARTICLE

Mass spectrometry-assisted identification of ADAMTS13-derived peptides presented on HLA-DR and HLA-DQ

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ABSTRACT

Formation of microthrombi is a hallmark of acquired thrombotic thrombocytopenic purpura. These microthrombi originate from insufficient processing of ultra large von Willebrand factor multimers by ADAMTS13 due to the development of anti-ADAMTS13 autoantibodies. Several studies have identified the major histocompatibility complex class II alleles HLA-DRB1*11, HLA-DQB1*03 and HLA-DQB1*02:02 as risk factors for acquired thrombotic thrombocytopenic purpura development. Previous research in our department indicated that ADAMTS13 CUB2 domain-derived peptides FINVAPHAR and LIRDTHSLR are presented on HLA-DRB1*11 and HLA-DRB1*03, respectively. Here, we describe the repertoire of ADAMTS13 peptides presented on HLA-DQ. In parallel, the repertoire of ADAMTS13-derived peptides presented on HLA-DR was monitored. Using HLA-DR- and HLA-DQ-specific antibodies, we purified HLA/peptide complexes from ADAMTS13-pulsed monocyte-derived dendritic cells. Using this approach, we identified ADAMTS13-derived peptides presented on HLA-DR for all 9 samples analyzed; ADAMTS13-derived peptides presented on HLA-DQ were identified in 4 out of 9 samples. We were able to confirm the presentation of the CUB2 domain-derived peptides FINVAPHAR and LIRDTHSLR on HLA-DR. In total, 12 different core-peptide sequences were identified on HLA-DR and 8 on HLA-DQ. For HLA-DR11, several potential new core-peptides were found; 4 novel core-peptides were exclusively identified on HLA-DQ. Furthermore, an *in silico* analysis was performed using the EpiMatrix and JanusMatrix tools to evaluate the eluted peptides, in the context of HLA-DR, for putative effector or regulatory T-cell responses at the population level. The results from this study provide a basis for the identification of immuno-dominant epitopes on ADAMTS13 involved in the onset of acquired thrombotic thrombocytopenic purpura.

Introduction

Thrombotic thrombocytopenic purpura (TTP) is a severe life-threatening disorder caused by decreased levels of functional ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin type 1 motifs, member 13). In healthy individuals, ADAMTS13 regulates the size of von Willebrand Factor (VWF) multi-

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mers through cleavage of a Tyr1605-Met1606 peptide bond in the A2 domain of VWF.[1,2] Functional or quantitative defects in ADAMTS13 levels in the circulation lead to the accumulation of high molecular weight VWF multimers and the formation of platelet- and VWF-rich thrombi. Within the microvasculature, these thrombi cause mechanical fragmentation of erythrocytes inducing hemolytic anemia.[1,2] In addition, the presence of hyperadhesive VWF multimers results in platelet consumption. As a consequence, patients with TTP often present with skin petechiae due to thrombocytopenia-induced blood loss from small vessels in the skin.[1,3] Additional clinical symptoms may include fever, renal failure or neurological abnormalities.[1,2]

In the majority of patients with TTP, the decrease in ADAMTS13 levels is due to the development of autoantibodies directed towards ADAMTS13. Most of these autoantibodies are composed of IgG1 and IgG4 subclasses;[4-6] these antibodies either inhibit the proteolytic function of ADAMTS13 or enhance its clearance from the circulation.[6-9] While the mechanisms responsible for the development of anti-ADAMTS13 antibodies are currently unknown, several reports have suggested that infections, pregnancy or transplantation may be considered to be risk factors for the onset of acquired TTP.[10-12] The generation of high affinity antibodies against ADAMTS13 is dependent on the help of specific $CD4^+$ T cells. Priming of antigen-specific $CD4^+$ T cells requires presentation of ADAMTS13-derived peptides on major histocompatibility complex class II (MHC-II) on professional antigen presenting cells.[13] The MHC-II genes are highly polymorphic allowing for the selection of a broad repertoire of $CD4^+$ T cells that is needed to combat infections. Specific MHC-II alleles have been linked to autoimmune disorders such as rheumatoid arthritis and celiac disease.[14] Similarly, association studies from three different cohorts of patients with acquired TTP have identified HLA-DRB1*11 as a risk factor.[15-17] Conversely, the frequency of HLA-DRB1*04 was significantly lower in patients with acquired TTP, suggesting a protective effect of this allele.[15-17] In addition to HLA-DRB1*11, higher frequencies of alleles HLA-DQB1*03[15,16] and HLA-DQB1*02:02[17] were found in patients with acquired TTP when compared to healthy controls. A recent study of 190 Italian TTP patients and 1255 healthy controls suggested that HLA-DQB1*05:03 was less prevalent in patients with acquired TTP.[18] This study also proposed that the common single nucleotide polymorphism rs6903608, which is located between the genes encoding the alpha and beta5 chains of the HLA-DR complex, combined with HLA-DQB1*05:03 explains most of the observed association between the HLA locus and acquired TTP.[18] As yet, the molecular mechanism underlying the observed association between polymorphic sites within the MHC II locus and acquired TTP has not been identified.

Previous observation from our laboratory has shown that monocyte-derived dendritic cells (mo-DCs) from healthy donors preferentially presented two peptides derived from the CUB2 domain of ADAMTS13.[19] Both of these peptides were found to activate $CD4^+$ T cells of patients with acquired TTP.[20] In addition, CUB2 domain-derived peptide $ADAMTS13^{1239\text{-}1253}$ was identified as an immunodominant T-cell epitope in an HLA-DRB1 transgenic mouse model.[21] The same study revealed that $ADAMTS13^{1239\text{-}1253}$ reactive $CD4^+$ T cells were present in patients with acquired TTP as well as in peripheral blood of healthy individuals.[21] As yet, the presentation of ADAMTS13-derived peptides on HLA-DQ has not been investigated. In the present work, we aimed to define the repertoire of ADAMTS13-derived peptides presented on HLA-DQ and prospectively identify putative effector and tolerated/tolerogenic T-cell epitopes using computational tools (EpiMatrix and JanusMatrix).

Methods

Materials

Recombinant full length ADAMTS13 was produced in stable transfected HEK293 cells and purified as described previously.[9] Concentration of purified ADAMTS13 was determined using the Bradford assay. Lipopolysaccharide (LPS) was obtained from Sigma-Aldrich (St. Louis, USA). The hybridoma producing the HLA-DQ-specific antibody (SPV-L3)[22] was a kind gift from Prof. dr. H. Spits (Academic Medical Center, Amsterdam, the Netherlands). The hybridoma producing the HLA-DR-specific monoclonal antibody (L243) was purchased from ATCC (Wesel, Germany). Antibodies were purified from culture supernatant *via* protein A Sepharose (GE Healthcare) and coupled to CNBr Sepharose 4B at a final concentration of 2 mg/mL (Amersham Biosciences, Buckinghamshire, UK).

Endocytosis of ADAMTS13 and affinity purification of HLA-DR and HLA-DQ

Monocytes were obtained from healthy volunteers in accordance with Dutch regulations after approval from the Sanquin Ethical Advisory Board, in accordance with the Declaration of Helsinki. After five days of differentiation, $5x10^6$ immature mo-DCs were incubated with 100 nM of recombinant ADAMTS13 in Cellgro medium supplemented with 800 U/ml IL-4 and 1000 U/mL GM-CSF. After five hours of incubation, the medium was supplemented without washing with 1 μg/mL of LPS and 1% fetal calf serum to allow for mo-DCs maturation overnight. After maturation, the cells were detached from the plate using trisodium citrate and gentle pipetting. HLA-DR and HLA-DQ peptide complexes were purified using a modification of a previously described protocol.[23] Briefly, cells were resuspended in 500 μL lysis buffer containing 10 mM Tris-HCl (pH 8.0), 0.25% octyl-β-D-glucopyranoside, 1% sodium deoxycholate and Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific), and incubated at 4°C for 30 minutes (mins). The lysed cells were centrifuged at 20,000xg for 15 mins at 4°C. HLA-DR and HLA-DQ peptide complexes were purified from the supernatant by adding L243 or SPV-L3 coupled CNBr Sepharose, respectively, and incubating the tubes at 4°C overnight 'end-over-end', using a laboratory rotator. L243 or SPV-L3 Sepharose beads were then washed 2 times with lysis buffer and 5 times with 10 mM Tris-HCl (pH 8.0). HLA-peptide complexes were eluted using 500 μL 10% acetic acid for 10 mins at room temperature. Then the samples were centrifuged at 400xg for five mins at room temperature and the supernatant transferred to low-binding 1.5 mL Eppendorf tubes and heated for 15 mins at 70°C. Samples were desalted using C18 STAGE Tips (Dr. Maisch Gmbh, Amersfoort, the Netherlands).[24] STAGE Tips were eluted with 60 μL of 1% formic acid/30% acetonitrile and eluates were concentrated using a SpeedVac (Savant, SPP1110, Thermo Scientific) to a final volume of 5 μL.

Mass spectrometry data analysis

Raw data files from the Orbitrap Fusion Tribid were scored against the uniprot-organism_9606_AND_keyword_kw_0181

Table 1. MHC-II genotype of healthy donors included in the study. The donors included in this study were typed for HLA-DRB1/DQB1 using PCR-SBT and HLA-DQA1 using next generation sequencing workflow.

Donor	HLA-DRB1		HLA-DQA1		HLA-DQB1	
1	07:01	14:54	01:04	02:01	03:03	05:03
2	04:04	15:01	01:02	03:01	03:02	06:02
3	01:01	15:01	01:01	04:01	05:01	06:02
4	01:01	08:01	01:01	04:01	04:02	05:01
5	01:01	07:01	01:01	02:01	02:02	05:01
6	01:01	11:01	01:01	05:05	03:01	05:01
7	01:01	11:01	01:01	05:05	03:01	05:01
8	04:01	11:01	03:01	05:05	03:01	03:02
9	03:01	13:01	01:01	05:01	02:01	06:03

database using Proteome Discover 1.4 (Thermo Scientific) with a 20 ppm tolerance for precursor mass and 10 ppm tolerance for fragment mass. Oxidation (+15.995 Da) on methionine was selected as dynamic modification. A decoy database comprising the reverse protein sequences from the same database was used to obtain a false discovery rate (FDR). Only peptides with a high or medium confidence (FDR threshold 0.05%) were considered for protein scoring.

Results

Presentation of ADAMTS13-derived peptides on HLA-DQ

To assess the contribution of HLA-DQ to the presentation of ADAMTS13-derived peptides, we pulsed mo-DCs from a panel of 9 HLA-typed healthy donors with 100 nM ADAMTS13. The HLA type of the donors included in this study is shown in Table 1. Mo-DCs from the same panel of donors were recently analyzed for the presentation of FVIII-derived peptides on HLA-DQ.[25] Mo-DCs were incubated with 1 μg/mL LPS and 1% fetal calf serum to allow for their maturation. HLA-DR and HLA-DQ molecules were purified employing monoclonal antibodies directed against HLA-DR (L243) and HLA-DQ (SPV-L3),[22] and the eluates were analyzed by mass-spectrometry. In agreement with our previous reports,[19,23,25] peptides derived from endogenously expressed as well as internalized proteins were presented on HLA-DR and HLA-DQ (*Online Supplementary Table S1A and B*, respectively). A subset of peptides was derived from proteins that are found within the endolysosomal compartment; these include HLA-DR itself, several proteases (such as cathepsin B) and endocytic receptors (such as the macrophage mannose receptor and prolow-density lipoprotein receptor-related protein 1).

The total number of unique peptides that was identified in the pulldowns of HLA-DR and HLA-DQ is shown in Figure 1A and *Online Supplementary Figure S1*. The total number of peptides presented on HLA-DQ was approximately 3-fold lower when compared to HLA-DR (median of 566 unique peptides on HLA-DQ compared to 1521 on HLA-DR). Similarly, although not statistically significant, the number of ADAMTS13-derived peptides presented on HLA-DQ was 3-fold lower when compared to HLA-DR (Figure 1B). HLA-DR-presented ADAMTS13-derived peptides were identified for all donors whereas ADAMTS13-derived peptides presented on HLA-DQ were only found in 4 out of 9 donors (Table 2A and B, and *Online Supplementary Figure S1B*).

Repertoire of ADAMTS13-derived peptides presented on HLA-DR

In order to compare the repertoire of ADAMTS13-derived peptides presented on HLA-DR and HLA-DQ, the binding cores of the ADAMTS13-derived unique peptides were predicted using the NetMHCIIpan 3.1 software (Table 2A and B).[26] ADAMTS13-derived peptides that shared the same core sequence were grouped (Figure 2). Previously, we have shown that ADAMTS13-derived peptides containing the sequence FINVAPHAR were presented on HLA-DRB1*11.[19] Peptides sharing the same sequence were also presented on non-HLA-DRB1*11 positive donors when mo-DCs were pulsed with 500 nM of ADAMTS13.[19] In the current study, peptides containing the FINVAPHAR sequence were presented on mo-DCs of HLA-DRB1*11 positive donors 7 and 8. Peptides with core-sequence FINVAPHAR were also presented by mo-DCs derived of HLA-DRB1*11 negative donors 1 and 9 (Table 2A). However, no peptides containing the FINVAPHAR sequence were identified in HLA-DRB1*11 positive donor 6 (Table 2A). Peptides derived from the sequence IHALATNMG which is located adjacent to the FINVAPHAR peptide were found in DRB1*11 negative donor 2 (Table 2A). Peptides derived from sequence LIRDTHSLR were identified in DRB1*03 positive donor 9. In a previous study, LIRDTHSLR-derived peptides were also presented by mo-DCs derived of DRB1*03 positive donors.[19] It is noteworthy that donors 6 and 7 with the same HLA-DRB1 haplotype (DRB1*01:01/DRB1*11:01) both presented peptides derived of core sequence LKTLPPARC originating from the TSP3 domain of ADAMTS13 (Table 2A). The majority of the HLA-DR-presented peptides identified in this study were derived from the CUB domains (Table 2A and Figure 2).

HLA-DQ contributes to presentation of ADAMTS13-derived peptides

We found ADAMTS13-derived peptides associated with HLA-DQ in 4 out of 9 donors (Table 2B and Figure 2). We identified 4 sets of peptides that were exclusively presented by HLA-DQ that originated from the cysteine-rich, TSP2, and TSP2-linker 1 of ADAMTS13 (Figure 2). This suggests that the repertoire of peptides presented on HLA-DQ differs from that presented on HLA-DR. We also identified peptides that were presented both on HLA-DR

and HLA-DQ. This is exemplified by the peptide derived from the CUB1 domain of ADAMTS13, spanning amino acid sequence 1206-1222 – RGPGQADCAVAIGRPLG, that was identified on both HLA-DR and HLA-DQ of donors 2 and 3 (Figure 2). However, in the case of donor 9 this peptide was found on HLA-DQ but not on HLA-DR. Similarly, two peptides derived from the metalloprotease and CUB2 domains of ADAMTS13 were identified both on HLA-DR and HLA-DQ for donors 8 and 9, respectively.

Our data show that, while HLA-DQ is involved in ADAMTS13, HLA-DR is more likely to be presenting peptides from ADAMTS13, and that there is considerable overlap between the repertoires of ADAMTS13-derived peptides presented on HLA-DR and HLA-DQ.

Evaluation of ADAMTS13 peptide immunogenicity using bioinformatic approaches

The above findings document which ADAMTS13 pep-

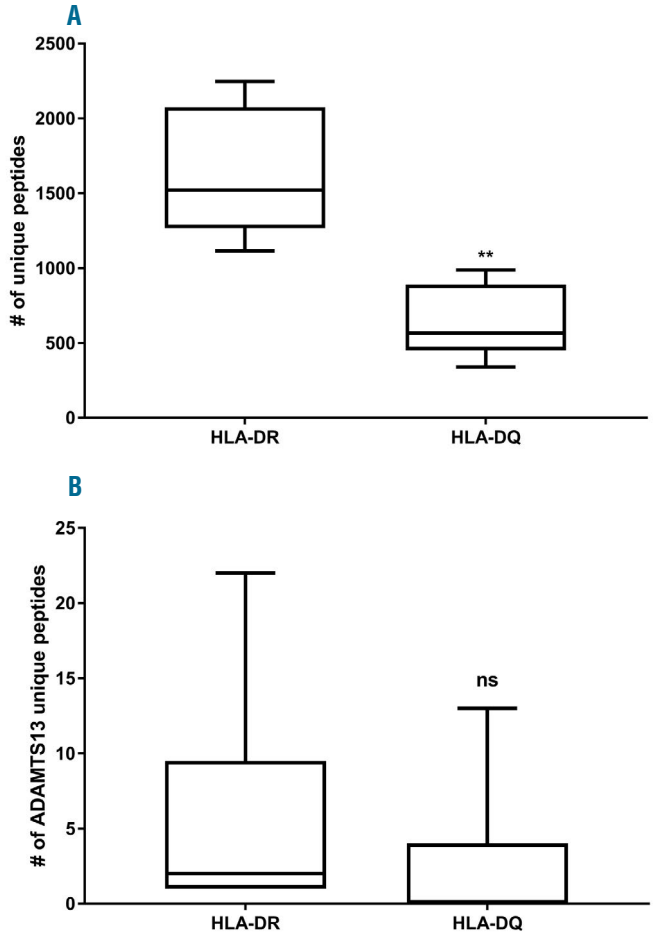

Figure 1. Relative presentation of total peptides and ADAMTS13-derived peptides on HLA-DR and HLA-DQ. (A) Total number of peptides identified after elution from either HLA-DR or HLA-DQ. (B) The ADAMTS13-derived peptides found on either HLA-DR or HLA-DQ. Statistical differences in peptide presentation were determined using the non-parametric Mann-Whitney *U*-test. ***P*<0.01. ns: not significant.

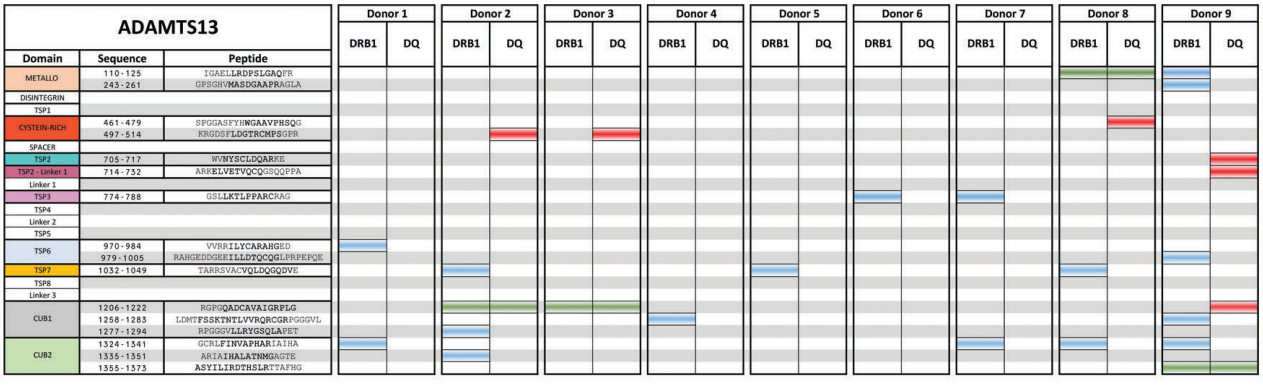

Figure 2. ADAMTS13-derived peptides presented on HLA-DR and HLA-DQ. Peptides identified to be presented by DCs of 9 studied donors on HLA-DR and HLA-DQ. Longest amino acid sequence of overlapping peptides is shown in the third column. Amino acids that are predicted to be a part of the peptide-MHC-II binding core are shown in bold. Blue: peptides identified only in HLA-DR condition. Red: peptides identified exclusively in HLA-DQ condition. Green: peptides identified in both HLA-DR and HLA-DQ conditions.

Table 2. HLA-DRB1 and HLA-DQA1/DQB1 peptide presentation and core-peptide binding affinity prediction. The ADAMTS13-derived peptides identified for each donor were analyzed for their binding affinities to the donor specific HLA-DRB1 (A) or HLA-DQA1/HLA-DQB1 alleles (B) using the NetMHCIIpan 3.1 software. The affinity value is presented in nM. For each peptide, a binding core was predicted. Binding cores with the highest affinity to the particular MHC-II are shown in yellow. Right panel represents the domain origin of each peptide.

A

Donor	Peptide	Predicted affinity (nM)		Position	Length (aa)	Domain
		DRB1*07:01	DRB1*14:54			
1	VVRRILYCARAHGED			970-984	15	TSP6
	----ILYCARAHG--	145.80	78.18	974-982		
	GCRLFINVAPHARIA			1324-1338	15	CUB2
	GCRLFINVAPHAR--			1324-1336	13	
	----FINVAPHAR--	24.48	40.25	1328-1336		
		DRB1*04:04	DRB1*15:01			
2	TARRSVACVQLDQGQDVE			1032-1049	18	TSP7
	--RRSVACVQLDQGQD--			1034-1047	14	
	--------VQLDQGQDV-	1977.48	2907.32	1040-1048		
	GPGQADCAVAIGRPL			1207-1221	15	CUB1
	------CAVAIGRPL	4810.42	1983.95	1213-1221		
	RPGGGVLLRYGSQLAPET			1277-1294	18	CUB1
	RPGGGVLLRYGSQLAPE-			1277-1293	17	
	------LLRYGSQLA---	209.79	175.16	1283-1291		
	ARIAIHALATNMGAGTE			1335-1351	17	CUB2
	ARIAIHALATNMGAGT-			1335-1350	16	
	-RIAIHALATNMGAGT-			1336-1350	15	
	-RIAIHALATNMGAG--			1336-1349	14	
	--IAIHALATNMGAGT-			1337-1350	14	
	--IHALATNMG------	40.19	145.33	1339-1347		
		DRB1*01:01	DRB1*15:01			
3	GPGQADCAVAIGRPL			1207-1221	15	CUB1
	------CAVAIGRPL	402.67	1983.95	1213-1221		
		DRB1*01:01	DRB1*08:01			
4	LDMTFSSKTNTLVVR			1258-1272	15	CUB1
	----FSSKTNTLV--	31.96	204.66	1262-1270		
		DRB1*01:01	DRB1*07:01			
5	ARRSVACVQLDQGQDVE			1033-1049	17	TSP7
	-------VQLDQGQDV-	1467.81	1869.23	1040-1048		
		DRB1*01:01	DRB1*11:01			
6	GSLLKTLPPARCRAG			774-788	15	TSP3
	---LKTLPPARC---	7.76	27.25	777-785		
		DRB1*01:01	DRB1*11:01			
7	GSLLKTLPPARCRAG			774-788	15	TSP3
	---LKTLPPARC---	7.76	27.25	777-785		
	RLFINVAPHARIA				13	CUB2
	--FINVAPHAR--	8.86	20.91	1328-1336		
		DRB1*04:01	DRB1*11:01			
8	IGAELLRDPSLGAQFR			110-125	16	METALLO
	IGAELLRDPSLGAQF-			110-124	15	
	IGAELLRDPSLGAQ--			110-123	14	
	IGAELLRD--------			110-117	8	
	----LLRDPSLGA---	106.23	637.43	114-122		
	RRSVACVQLDQGQDVE			1034-1049	16	TSP7
	------VQLDQGQDV-	2610.67	4300.29	1040-1048		
	RLFINVAPHARIA			1326-1338	13	CUB2
	RLFINVAPHAR--			1326-1336	11	
	--FINVAPHAR--	86.01	20.91	1328-1336		
		DRB1*03:01	DRB1*13:01			
9	IGAELLRDPSLGAQFR			110-125	16	METALLO
	IGAELLRDPSLGAQF-			110-124	15	
	IGAELLRDPSLGAQ--			110-123	14	
	----LLRDPSLGA---	34.36	637.43	114-122		
	GPSGHVMASDGAAPRAGLA			243-261	19	METALLO
	GPSGHVMASDGAAPRAG--			243-259	17	
	------MASDGAAPR----	301.23	3452.93	249-257		
	RAHGEDDGEEILLDTQCQGLPRPEPQE			979-1005	27	TSP6
	RAHGEDDGEEILLDTQCQGLPRPEPQ-			979-1004	26	
	------DGEEILLDTQCQGLPR-----			985-1000	16	
	--------EEILLDTQCQGLPR-----			987-1000	14	
	----------ILLDTQCQG--------	214.80	3088.83	989-997		
	TNTLVVRQRCGRPGGGVL			1266-1283	18	CUB1
	---LVVRQRCGR------	1918.45	620.69	1269-1277		
	LFINVAPHARIAIHA			1327-1341	15	CUB2
	-FINVAPHARIAIHA			1328-1341	14	
	--INVAPHARIAIHA			1329-1341	13	
	-FINVAPHAR-----	96.65	26.32	1328-1336		
	ASYILIRDTHSLRTTAFHG			1355-1373	19	CUB2
	ASYILIRDTHSLRTTAFH-			1355-1372	18	
	ASYILIRDTHSLRTTAF--			1355-1371	17	
	ASYILIRDTHSLRTTA---			1355-1370	16	
	ASYILIRDTHSLRT-----			1355-1368	14	
	-SYILIRDTHSLRTTA---			1356-1370	15	
	-SYILIRDTHSLRTT----			1356-1369	14	
	-SYILIRDTHSLRT-----			1356-1368	13	
	--YILIRDTHSLRT-----			1357-1368	12	
	----LIRDTHSLR------	12.73	43.39	1359-1367		

B

Donor	Peptide	Predicted affinity (nM)				Position	Length (aa)	Domain
		DQA1*01:02-03:01		DQB1*03:02-06:02				
		DQA1*01:02/DQB1*03:02	DQA1*01:02/DQB1*06:02	DQA1*03:01/DQB1*03:02	DQA1*03:01/DQB1*06:02			
2	GDSFLDGTRCMPSGPR					499-514	16	CYSTEIN-RICH
	-DSFLDGTRCMPSGPR					500-514	15	
	----LDGTRCMPS---	3153.77	1960.07	8303.76	2677.36	503-511		
	GPGQADCAVAIGRPL					1207-1221	15	CUB1
	---QADCAVAIG---	862.36	499.96	2763.65	634.30	1210-1218		
		DQA1*01:01-04:01		DQB1*05:01-06:02				
		DQA1*01:01/DQB1*05:01	DQA1*01:01/DQB1*06:02	DQA1*04:01/DQB1*05:01	DQA1*04:01/DQB1*06:02			
3	KRGDSFLDGTRCMPSGPR					497-514	18	CYSTEIN-RICH
	--GDSFLDGTRCMPSGPR					499-514	16	
	---DSFLDGTRCMPSGPR					500-514	15	
	------LDGTRCMPS---	6143.19	1907.21	3635.9	1881.12	503-511		
	GPGQADCAVAIGRPL					1207-1221	15	CUB1
	GPGQADCAVAIGRP-					1207-1220	14	
	---QADCAVAIG---	14273.44	602.38	5518.16	260.17	1210-1218		
		DQA1*03:01-05:05		DQB1*03:01-03:02				
		DQA1*03:01/DQB1*03:01	DQA1*03:01/DQB1*03:02	DQA1*05:05/DQB1*03:01	DQA1*04:01/DQB1*03:02			
8	IGAELLRDPSLGAQFR					110-125	16	METALLO
	-----LRDPSLGAQ--	1150.67	3011.83	661.33	670.73	115-123		
	SPGGASFYHWGAAVPHSQG					461-479	19	CYSTEIN-RICH
	---------WGAAVPHSQ-	73.84	745.00	13.72	41.71	470-478		
		DQA1*01:03-05:01		DQB1*02:01-06:03				
		DQA1*01:03/DQB1*02:01	DQA1*01:03/DQB1*06:03	DQA1*05:01/DQB1*02:01	DQA1*05:01/DQB1*06:03			
9	WVNYSCLDQARKE					705-717	13	TSP2
	WVNYSCLDQARK-					705-716	12	
	--NYSCLDQAR--	4586.78	937.29	3601.95	586.21	707-715		
	ARKELVETVQCQGSQQPPA					714-732	19	TSP2 - Linker 1
	-RKELVETVQCQGSQQPPA					715-732	18	
	--KELVETVQCQGSQQPP-					716-731	16	
	---ELVETVQCQ-------	2320,32	341.78	1195.46	260.77	717-725		
	RGPGQADCAVAIGRPLG					1206-1222	17	CUB1
	-GPGQADCAVAIGRPLG					1207-1222	16	
	-GPGQADCAVAIGRPL-					1207-1221	15	
	-------CAVAIGRPL-	9282.49	275.81	5222.25	50.21	1213-1221		
	ASYILIRDTHSLRTTA					1355-1370	16	CUB2
	ASYILIRDTHSLRT--					1355-1368	14	
	-SYILIRDTHSLRTTA					1356-1370	15	
	-SYILIRDTHSLRTT-					1356-1369	14	
	--YILIRDTHSLRT--					1357-1368	12	
	ASYILIRDT-------	2888.92	537.15	3437.09	421.72	1355-1363		

tides are displayed on MHC class II following pulsing of dendritic cells with ADAMTS13. To complement our analysis of peptides eluted from MHC, we employed a bio-informatic approach to identify whether the ADAMTS13-derived peptides identified in this and a previous study (Table 3)[19] might be immunogenic. Firstly, we used the EpiMatrix algorithm, which assesses the potential for individual peptides to bind to HLA-DR based on amino acid motifs preferred by nine "supertype" HLA-DR allele families. These HLA-DR motif families cover 95% of the human population worldwide.[27] EpiMatrix assesses peptides for binding potential against a matrix of amino acid preferences for each HLA allele. This score is adjusted to a normalized (Z) score. The EpiMatrix Score of a peptide represents a sum of significant assessments for all of the nine-mers in a sequence, adjusted for length. An EpiMatrix Score of 40.66 was obtained for metallo-protease-derived peptide SRRQLLSLLSAGRAR (residues 266-280); this value indicates that this peptide has a significant potential to bind to multiple HLA-DR molecules (Table 3). Also, the CUB2 domain-derived peptide FSEGFLKAQASLRGQYW (residues 1390-1406) was predicted to bind to multiple HLA-DR alleles (Table 3). A number of other CUB1-2 domain-derived peptides were found to be potentially immunogenic, as revealed by an EpiMatrix Score higher than 10 (Table 3).

A second tool, JanusMatrix,[28] was used to evaluate whether the HLA-DR-presented ADAMTS13 peptides displayed homology to proteins from the human proteome. JanusMatrix differentiates HLA-binding peptides that are more cross-conserved at the TCR face and thus more likely tolerated (or actively tolerogenic) from those less cross-conserved that are more likely to be immunogenic.[29,30] This analysis revealed that six of the peptides (Table 3 and

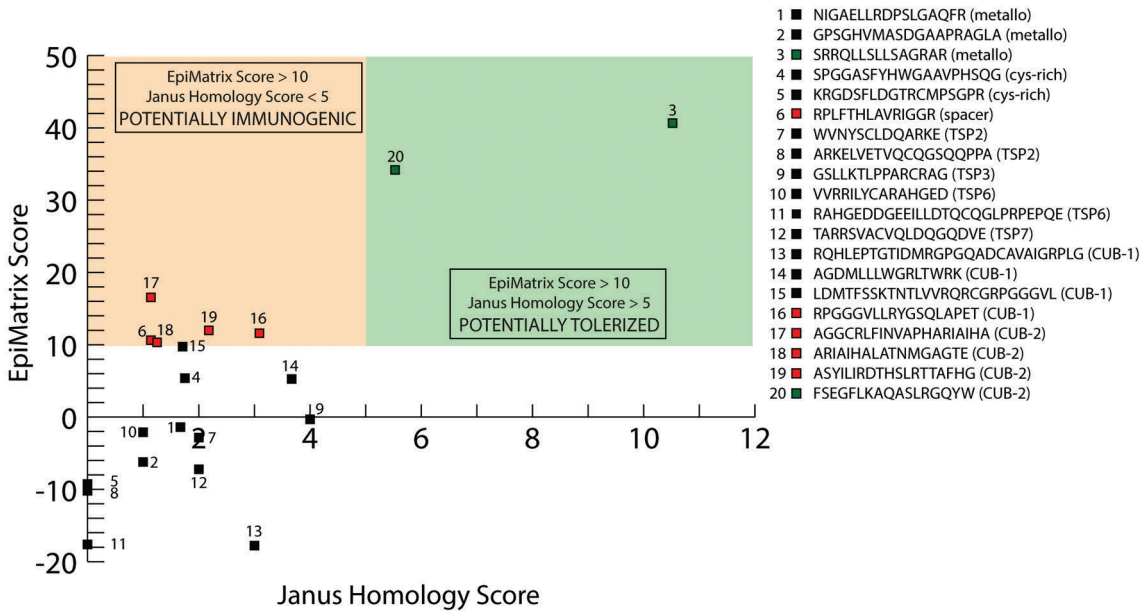

Figure 3. Schematic representation of eluted peptide immunogenicity. EpiMatrix and JanusMatrix Homology Scores for each eluted peptide is depicted in the graph. In the context of nine population-spanning "supertype" HLA-DR alleles, peptides with high (>10, orange) EpiMatrix Score are predicted to be promiscuous epitopes, presented by multiple HLA-DR alleles. Additionally, peptides with elevated to high (>3) JanusMatrix Homology Scores display high homology with human proteome at the TCR face and are predicted to be tolerated or actively tolerogenic (green squares). Peptides with high EpiMatrix Score (>10) in combination with low JanusMatrix Homology Score (<3) are predicted to be immunogenic (red squares). All other peptides, with an EpiMatrix Score <10, have limited or HLA-restricted immunogenic potential (black squares).

Figure 3) showed promiscuity in binding to supertype HLA-DR alleles in combination with low cross-conservation with human proteins. These peptides could potentially initiate an effector T-cell response. In contrast, four of the peptides (Table 3 and Figure 3) were found to be cross-conserved within the human protein repertoire and could thus be potentially tolerated or actively tolerogenic in patients with acquired TTP. The remaining 10 ADAMTS13 peptides did not register high EpiMatrix Scores for binding to HLA-DR. However, they did have significant assessments for individual HLA-DR alleles and therefore could be presented on a subset of MHC II molecules.

Discussion

In this study, we explored the repertoire of HLA-DQ-presented peptides on ADAMTS13 pulsed monocyte-derived dendritic cells. In parallel, we also assessed the HLA-DR-presented peptide repertoire. This approach allows for a direct comparison of the peptide repertoires presented on HLA-DR and HLA-DQ. For this, we used two different monoclonal antibodies, L243 and SPV-L3, that had been previously used for peptide presentation profiling on either HLA-DR or HLA-DQ.[19,23,31-33] Our data revealed that the number of peptides presented on HLA-DQ is 2- to 3-fold lower when compared to the repertoire presented on HLA-DR. This is most likely explained by the higher expression of HLA-DR on dendritic cells when compared to HLA-DQ.[25,34,35] Furthermore, this could also result from an overall lower binding affinity of peptides for HLA-DQ when compared to HLA-DR. Indeed, the NetMHCIIpan3.1 prediction tool suggests a higher binding affinity of ADAMTS13-derived peptides to HLA-DR when compared to HLA-DQ. In a recent study, we also observed preferential presentation of blood coagulation FVIII-derived peptides on HLA-DR when compared to HLA-DQ.[25]

Interestingly, using higher-energy collisional dissociation fragmentation, the total number of peptides identified in HLA-DR eluates ranged from 1115 to 2247. This represents a 5- to 6-fold increase when compared to a previous study from our group that employed collision-induced dissociation.[19] We anticipate that the use of a highly sensitive mass spectrometer (Orbitrap Fusion Tribrid) has allowed for an increased number of peptides identified on HLA-DR. Surprisingly, despite the use of a highly sensitive mass-spectrometry strategy, the number of ADAMTS13-derived peptides remained relatively low in the case of HLA-DR and even lower for HLA-DQ (an average of 7 *vs.* 3 ADAMTS13-derived peptides, respectively). In the current study, we pulsed mo-DCs with 100 nM of recombinant ADAMTS13, whereas circulating levels of ADAMTS13 in healthy individuals ranges from 3.5-7 nM (740-1420 ng/mL).[36] Previous work from our group has identified the mannose receptor as an endocytic receptor for ADAMTS13 by mo-DCs.[37] More recently, we described the involvement of CD163 in the internalization of ADAMTS13 by macrophages *in vitro*.[38] In the latter study, we observed that the uptake efficiency of CD163-positive macrophages was approximately 10 times higher when compared to that of mo-DCs (that are devoid of CD163 expression). Based on this finding, it is tempting to speculate that the limited presentation of ADAMTS13-derived peptides on mo-DCs is due to a relatively low efficiency to internalize ADAMTS13, thereby limiting the number of ADAMTS13-derived peptides that are presented. Whether the limited presentation of ADAMTS13 by dendritic cells explains the low incidence of TTP in the general population despite the high prevalence of the risk

Table 3. Evaluation of ADAMTS13 peptide immunogenicity *in silico*.

Peptide sequence	Domain of ADAMTS13	Position	Length	EpiMatrix Score	Number of human matches	Janus Homology Score
NIGAELLRDPSLGAQFR	Metallo	109-125	17	-1.40	2	1.67
GPSGHVMASDGAAPRAGLA	Metallo	243-61	19	-6.22	2	1.00
SRRQLLSLLSAGRAR	Metallo	266-280	15	40.66	90	10.52
SPGGASFYHWGAAVPHSQG	Cystein-rich	461-79	19	5.38	5	1.75
KRGDSFLDGTRCMPSGPR	Cystein-rich	497-514	18	-9.27	0	n/a
RPLFTHLAVRIGGR	Spacer	589-602	14	10.64	4	1.14
WVNYSCLDQARKE	TSP2	705-717	13	-2.84	2	2.00
ARKELVETVQCQGSQQPPA	TSP2	714-732	19	-10.20	0	n/a
GSLLKTLPPARCRAG	TSP3	774-788	15	-0.32	8	4.00
VVRRILYCARAHGED	TSP6	970-984	15	-2.10	2	1.00
RAHGEDDGEEILLDTQCQGLPRPEPQE	TSP6	979-1005	27	-17.61	0	n/a
TARRSVACVQLDQGQDVE	TSP7	1032-1049	18	-7.23	2	2.00
RQHLEPTGTIDMRGPGQADCAVAIGRPLG	CUB-1	1194-1222	29	-17.79	3	3.00
AGDMLLLWGRLTWRK	CUB-1	1238-1252	15	5.27	14	3.67
LDMTFSSKTNTLVVRQRCGRPGGGVL	CUB-1	1258-1283	26	9.75	13	1.71
RPGGGVLLRYGSQLAPET	CUB-1	1277-1294	18	11.60	16	3.09
AGGCRLFINVAPHARIAIHA	CUB-2	1322-1341	20	16.56	7	1.14
ARIAIHALATNMGAGTE	CUB-2	1335-1351	17	10.33	5	1.25
ASYILIRDTHSLRTTAFHG	CUB-2	1355-1373	19	12.00	12	2.18
FSEGFLKAQASLRGQYW	CUB-2	1390-1406	17	34.19	29	5.53

Binding of peptides to nine common HLA-DR allele families was predicted using the EpiMatrix algorithm developed by EpiVax. An EpiMatrix Score that reflects peptide binding potential to each of the 9 HLA-DR alleles was assigned to each peptide. Peptides with EpiMatrix scores > 5 are predicted to have elevated immunogenic potential; peptides with scores > 10 are predicted to have significant immunogenic potential. In addition, JanusMatrix was used to predict potential cross-reactivity between the peptides and the human proteome, based on conservation of TCR-facing residues. A JanusMatrix Homology Score was assigned to each peptide. Higher scores indicate greater conservation with the human proteome. At the population level, given elevated to high EpiMatrix scores, peptides with low cross-conservation with human proteome (scores < 3) are considered likely to be immunogenic, while peptides with scores > 3 have elevated to high cross-conservation with human proteome and are thus potentially tolerated or tolerogenic.

alleles HLA-DRB1*11 and DQB1*03 in the general population represents an interesting question that needs further study.

The overall low number of ADAMTS13-derived peptides reported may also be due to post-translational modifications of HLA-DR/DQ-presented peptides that interfere with their identification. For instance, the presence of a glycan results in a shift in the net mass of the glycan-bearing peptide, impairing its identification by mass spectrometry. Ten N-glycosylation sites, 8 O-fucosylation sites and 3 C-mannosylation sites have been described for plasma-derived ADAMTS13.[39,40] As yet, the impact of post-translational modifications such as N-linked glycosylation on MHC-II peptide presentation has not been extensively studied. To our surprise, we also identified a peptide derived from the TSP2 domain of ADAMTS13 (NYSCLDQAR) that has been reported to contain an N-linked glycosylation site.[39] A recent publication from our group showed that the asparagine present within this peptide contains a bi-antennary complex-glycan.[40] As mentioned earlier, the presence of a glycan interferes with the identification of the glycan-bearing peptide by mass spectrometry. However, it was previously described that the third position in peptides with consensus sequence Asn-X-Ser/Thr affects glycosylation efficiency, Thr being associated with a higher degree of glycosylation compared to Ser.[41] It is, therefore, likely that the identified peptide NYSCLDQAR is partially glycosylated, allowing for the identification of the non-glycosylated version in our study. Whether the glycosylated counterpart of this peptide is also presented on HLA molecules remains to be determined. How the presence or absence of an N-linked glycan at this position modulates peptide/MHC-II/T-cell receptor (TCR) binding represents an interesting question. On one hand, the presence of an N-linked glycan at this position could interfere with the binding of the peptides to HLA-DQ or with the recognition of the HLA-DQ/peptide complex by a complementary T-cell receptor (TCR). Interestingly, absence of an O-linked glycan on type II collagen has been previously suggested to create a novel T-cell epitope that has been implicated in the development of autoimmune arthritis.[42-44] Similarly, $CD4^+$ T cells recognizing non-glycosylated forms of ADAMTS13-derived peptides that normally contain a glycan may contribute to the onset of autoimmune TTP. On the other hand, the presence of a glycan can also lead to the formation of a neo-epitope.[45] In view of the large number of N-and O-linked glycans on ADAMTS13, it cannot be excluded that

glycan-containing peptides derived of ADAMTS13 are presented on MHC-II.[40]

Identification of the immunogenic determinants of ADAMTS13 represents a major challenge for the understanding of TTP pathogenesis. Our study shows that there is an overlap, but also differences, in the repertoire of ADAMTS13-derived peptides that are presented on HLA-DQ and HLA-DR. This is illustrated by the peptide with core sequence FINVAPHAR derived from the CUB2 domain that was identified exclusively in the case of HLA-DR. In contrast with the study of Sorvillo *et al*., only 2 out of the 3 HLA-DRB1*11 donors presented the FINVAPHAR peptide. In addition, this peptide was also identified in 2 non-HLA-DRB1*11 donors, confirming that the presentation of FINVAPHAR is not restricted to HLA-DRB1*11.[19] Interestingly, several peptides were identified associated with HLA-DQ only. Especially, two peptides derived from the cysteine-rich domain were identified exclusively on HLA-DQ. Lastly, we identified sets of peptides that were presented on both HLA-DR and HLA-DQ, as exemplified by the CUB1-derived peptide with core-sequence CAVAIGRPL.

Whether the novel peptides identified in this study play a role in the onset of acquired TTP remains to be determined. A previous study of Verbij *et al*. showed that CUB2 domain-derived peptides FINVAPHAR and ASYILIRD are able to activate $CD4^+$ T cells from an HLA-DRB1*11 and an HLA-DRB1*03-positive acquired TTP patient, respectively.[20] A recent study by Gilardin *et al*. did not identify $CD4^+$ T cells responding to peptide $ADAMTS13^{1239\text{-}1253}$ containing the FINVAPHAR core-sequence in HLA-DRB1*11-positive patients.[21] In their hands, another CUB2 domain-derived peptide $ADAMTS13^{1239\text{-}1253}$ was identified as an immunodominant T-cell epitope for both DRB1*01 and DRB1*11-positive acquired TTP patients.[21] Surprisingly, the $ADAMTS13^{1239\text{-}1253}$ peptide was not identified to be presented on HLA-DR or DQ in our current study, despite the fact that several HLA-DRB1*01 and HLA-DRB1*11 positive donors were included in our cohort. Two peptides containing the same core-sequence were, however, identified in a DRB1*0401/DRB1*1301 positive donor in a previous study from our group.[19]

In silico approaches (EpiMatrix) identified a majority of ADAMTS13 peptides (70%) with significant EpiMatrix scores in the CUB1/2 domains. Six of the eluted ADAMTS13 peptides with low cross-conservation with the human proteome (based on JanusMatrix analysis) were considered to be more likely to elicit an effector T-cell response. These predicted immunogenic peptide sequences included $ADAMTS13^{1322\text{-}1341}$ (FINVAPHAR) and $ADAMTS13^{1355\text{-}1373}$ (ASYILIRD); $CD4^+$ T cells targeting these epitopes have indeed been found in patients with acquired TTP.[20] In contrast, four of the peptides had TCR facing residues that were highly conserved with multiple peptides found in the human proteome and putatively restricted by the same HLA, and, therefore, could potentially be tolerated or actively tolerogenic in patients with acquired TTP. These peptides include peptide $ADAMTS13^{1238\text{-}1252}$, containing a minimal core sequence recognized by the ADAMTS13-specific DRB1*01:01-restricted T-cell hybridomas as described by Gilardin *et al*.[21] $CD4^+$ T cells targeting different epitopes may develop in patients with acquired TTP. Phenotypic profiling of $CD4^+$ T-cell responses in large numbers of patients with acquired TTP will be needed to define whether multiple immune-dominant and potential immunoregulatory $CD4^+$ T-cell epitopes contribute to the etiology of acquired TTP.

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