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A Comparative Analysis of the Peptide Repertoires of HLA–DR Molecules Differentially Associated With Rheumatoid Arthritis

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Objective. **To evaluate similarity of the peptide repertoires bound to HLA–DR molecules that are differentially associated with rheumatoid arthritis (RA), and to define structural features of the shared peptides.**

Methods. **Peptide pools bound to HLA–DRB1*01:01, HLA–DRB1*04:01, and HLA–DRB1*10:01 (RA associated) and those bound to HLA–DRB1*15:01 (non–RA–associated) were purified and analyzed by liquid chromatography (LC) matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MS) and LC–ion-trap MS. Peptide pools from each allotype were compared in terms of size, protein origin, composition, and affinity (both theoretical and experimental with some peptides). Finally, 1 peptide sequenced from DR1, DR4, and DR10, but not from DR15, was modeled in complex with all 4 HLA–DRB1 molecules and HLA–DRB5*01:01.**

Results. **A total of 6,309 masses and 962 unique peptide sequences were compared. DR10 shared 29 peptides with DR1, 9 with DR4, and 1 with DR15; DR1 shared 6 peptides with DR4 and 9 with DR15; and DR4 and DR15 shared 4 peptides. The direct identification of peptide ligands indicated that DR1 and DR10 were the most similar molecules regarding the peptides that they could share. The peptides common to these molecules contained a high proportion of Leu at P4 and basic residues at P8 binding core positions.**

Conclusion. **The degree of overlap between peptide repertoires associated with different HLA–DR molecules is low. The repertoires associated with DR1 and DR10 have the highest similarity among the molecules analyzed (~10% overlap). Among the peptides shared between DR1 and DR10, a high proportion contained Leu[4] and basic residues at the P8 position of the binding core.**

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Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease affecting peripheral joints. The pathogenesis of the disease is complex, involving both environmental and genetic factors. The strongest genetic association is with the HLA–DRB1 locus (1–3), although other genes have also been related to RA, including *PTPN22*, *IL23R*, *TRAF1*, *CTLA4*, *IRF5*, *STAT4*, *CCR6*, *PADI4*, and others (1,4). HLA–DR molecules bind peptides mostly generated during the catabolism of proteins located in the endocytic pathway and present them to CD4+ T lymphocytes. Some HLA–DR alleles are positively associated with the disease, including some subtypes of HLA–DR4 (DRB1*04:01, DRB1*04:04, DRB1*04:05, DRB1*04:08), HLA–DR1 (DRB1*01:01, DRB1*01:02), and HLA–DR10 (DRB1*10:01), while other alleles are negatively associated with seropositive RA (e.g., HLA–DRB1*03:01, HLA–DRB1*07:01, HLA–DRB1*08:01) or are

considered non–RA-associated or low-risk alleles (e.g., HLA–DRB1*15:01) (2,5).

The RA-associated molecules have a consensus sequence spanning residues 70–74 of the HLA–DR β-chain containing basic residues. This feature led to the proposal of the "shared epitope" (SE) hypothesis (6), which postulates that this motif can modulate T cell recognition, either by directly interacting with the T cell receptor or by defining the HLA–DR–associated peptide repertoire through direct interaction with the peptides. One of the molecular contacts between the HLA–DR molecule and the peptide resides among residue 71 of the DRβ chain and the P4 position of the peptide binding core (7–9). The presence of basic residues in this position impairs the binding of basic residues, such as Arg, at the peptide P4 core position. However, RA-associated HLA–DR molecules allow the presence of Gln, which is similar to citrulline, at the P4 anchor position. The presence of citrullinated protein–specific antibodies in the serum of RA patients is closely linked to the expression of SE alleles (10,11).

HLA–DR4 is associated with RA in many populations, principally in Northern Europe (12), while DR1 and DR10 are predominant in RA patients in some populations of Southern Europe (13–15) and others (16–18). The peptide anchor motif of HLA–DRB1*10:01 was determined by our group through partial sequencing of its peptide repertoire (19). The anchor motif, with small differences, was subsequently confirmed by other techniques (20). Theoretically, there should be a higher degree of overlap between peptide repertoires bound by SE-containing HLA–DR allotypes, such as DRB1*01:01 and DRB1*04:01, than between repertoires bound to other low-risk or negatively associated molecules, such as DRB1*15:01 or DRB1*07:01 (19). Such repertoires could be inferred using bioinformatics-based prediction tools. However, the available tools use different algorithms and differ in the values that they assign to theoretical affinity. Therefore, direct sequencing of the peptide ligands of HLA–DR molecules is required to identify peptides that are bound to them and to confirm theoretical data. The identification of natural ligands of different alleles in samples processed in parallel could reveal structural features that allow some peptides to bind to SE-containing alleles but not to other RA-unrelated molecules.

HLA–DR molecules are promiscuous regarding the peptides they can bind (21,22). However, a direct quantification of the degree of overlap between different HLA–DR allotypes differentially associated with RA has not yet been performed. This approach has been successfully used to compare peptide pools sequenced from different HLA–B27 subtypes and to quantify the degree of overlap of these peptide repertoires (23–25). Herein we describe a comparison of peptide repertoires bound to HLA–DR alleles differentially associated with RA. Our analysis included 3 of the most clearly RA-associated alleles, HLA–DRB1*01:01, HLA–DRB1*04:01, and HLA–DRB1*10:01, and 1 non–RA-associated allele, HLA–DRB1*15:01. Homozygous lymphoblastoid cells were used as the source of the peptide–HLA–DR complexes, which were purified by immunoaffinity chromatography. After acid elution and ultrafiltration, peptides were analyzed by liquid chromatography matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (LC-MALDI-TOF-MS) and LC–ion-trap MS. Analysis of the peptide pools indicated relatively low overlap of the peptide repertoires of different HLA–DR molecules. The 2 most similar HLA–DR peptide repertoires were those from DR1 and DR10, which shared a high proportion of peptides with Leu at the P4 binding core position as well as a high proportion of basic residues at P8.

MATERIALS AND METHODS

Cell lines and antibody. Homozygous Epstein-Barr virus–transformed B-lymphoblastoid cell lines from the Tenth International Histocompatibility Workshop (26) included HOM-2 (DRA1*01:01/DRB1*01:01), MGAR (DRA1*01:01/DRB1*15:01), and WT51 (DRA1*01:01/DRB1*04:01). The HLA–DR10 homozygous B-lymphoblastoid cell line BEN (DRA1*01:01/DRB1*10:01) was previously described (19). The sequence alignment of the DRB1 alleles is shown in Supplementary Figure 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39736/abstract. Cells were grown in roller bottles in RPMI (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Invitrogen). Cells were washed 3 times in cold phosphate buffered saline, and dry pellets were stored at −70°C until processed. The HLA–DR–specific monoclonal antibody B8.11.2 (IgG2b) (27) was used for purification of peptide–HLA–DR complexes.

HLA–DR–associated peptide pool purification. Peptide–HLA–DR complexes were purified as described previously (19,28). Procedures are detailed in Supporting Information, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39736/abstract.

LC-MALDI-TOF/TOF-MS. Peptides were fragmented by reverse-phase high-performance LC (RP-HPLC). Fractions were obtained every 15 seconds and analyzed in a MALDI-TOF mass spectrometer (UltrafleXtreme; Bruker Daltonics). Peptide repertoires from the different allotypes were compared by pairs using the software MSHandler (29), setting the system as follows: the selected mass/charge (m/z) ratios ranged from 1,100 to 3,000; tolerances used to judge 2 mass signals as the equivalent peptide were set at $\pm$0.25 daltons/charge for the m/z value and $\pm$2 elution positions for the chromatographic retention time; the signal intensity cutoff for the whole spectrum was 100, in absolute value of intensity units, and the specific signal cutoff, discarding signals that were likely mistakenly assigned as specific because of lack of instrumental accuracy, was 500; the specific signals search width, discarding signals likely assigned

as specific due to a shift between both chromatographic profiles, was ±6 elution positions.

A false discovery rate (FDR) for the search method, depicting the probability that 2 different chromatographies show 2 equivalent masses corresponding to different peptides, was specifically calculated for each comparative analysis performed. FDR calculation was carried out by determining the overlap between the real sample chromatography and a fictional chromatography so-called decoy. The decoy was made by calculating the mass of a wide pool of randomly generated amino acid sequences containing 9–27 residues that were distributed along 250 theoretical chromatographic fractions, placing 20–30 masses in each fraction.

Ion-trap MS and sequence identification. Peptides were analyzed using a 3-dimensional ion-trap mass spectrometer (Esquire HCT Ultra; Bruker Daltonics) (19,30) and a high-resolution quadrupole/linear ion-trap mass spectrometer (LTQ-Orbitrap XL; Thermo Fisher) (30–32). Procedures are detailed in Supporting Information, http://onlinelibrary.wiley.com/doi/10.1002/art.39736/abstract.

Predicted binding affinity. The theoretical binding of each peptide to the different HLA–DR molecules was calculated using a NetMHCIIpan 3.1 Server (33). The 50% inhibition concentration (IC_{50}) value was considered. Peptides with an IC_{50} value lower than 50 n*M* were classified as strong binders, those with an IC_{50} value of 50–500 n*M* were classified as intermediate binders, and those with an IC_{50} value higher than 500 n*M* were classified as weak binders.

Peptide–HLA–DR binding assay. The experimental affinity of some peptides to HLA–DR molecules was assayed as previously described (31). Briefly, recombinant DR1 (DRA1*01:01/DRB1*01:01), DR4 (DRA1*01:01/DRB1*04:01), DR10 (DRA1*01:01/DRB1*10:01), DR2b (DRA1*01:01/DRB1*15:01), and DR2a (DRA1*01:01/DRB5*01:01) proteins were used for peptide binding assays. Increasing concentrations of each nonbiotinylated test peptide were incubated in competition with 0.02 m*M* biotinylated hemagglutinin 306–318 peptide ($HA^{306–318}$) in wells coated with HLA–DR1 or HLA–DR4 protein, 0.01 m*M* biotinylated $TT^{560–571}$ peptide in wells coated with HLA–DR10 protein, and 0.01 m*M* biotinylated modified glutamic acid decarboxylase$^{555–567}$ peptide (NLIRVVSSNRAAT, modified from NFFRMVISNPAAT to bind to DR1501 and DRB5 in a single unambiguous register) in wells coated with HLA–DR2a and HLA–DR2b protein. After washing, residual biotinylated reference peptide was labeled using europium-conjugated streptavidin (PerkinElmer) and quantified using a Victor^3 D time-resolved fluorometer (PerkinElmer). Peptide binding curves were simulated by nonlinear regression with GraphPad Prism software, version 4.03 using a sigmoidal dose-response curve. IC_{50} binding values were calculated from the resulting curves as the peptide concentration needed for 50% inhibition of reference peptide binding.

Modeling. Modeling of complexes between HLA–DR1 (DRB1*01:01), HLA–DR4 (DRB1*04:01), HLA–DR10 (DRB1*10:01), HLA–DR2b (DRB1*15:01), or HLA–DR2a (DRB5*01:01) and the peptide IVTERSVSS was performed using a simulation protocol introduced elsewhere (34) (see Supporting Information, http://onlinelibrary.wiley.com/doi/10.1002/art.39736/abstract). This procedure delivers a binding score that can be used to (crudely) estimate the relative binding affinity of different peptides for the same HLA molecule.

RESULTS

Overlap of the HLA–DR peptide repertoires. The HLA–DR peptide pools that were eluted from DR1, DR4, DR10, and DR15 were purified and analyzed by 2 different techniques: LC-MALDI-TOF/TOF-MS and LC–ion-trap MS. To evaluate the degree of overlap between the different peptide repertoires, the peptide pools were fragmented by RP-HPLC, and 240 fractions for each pool were collected and analyzed by MALDI-TOF/TOF-MS. A total of 6,309 signals with m/z of 1,100–3,000 were considered: 1,594 from DR1, 1,278 from DR4, 1,248 from DR10, and 2,189 from DR15. The peptide repertoires were compared by pairs. The most similar pools were those from DR1 and DR10, which shared 10.4% and 9.4%, respectively, of the masses analyzed (Table 1). These data confirmed the promiscuity of the peptides bound to HLA–DR molecules, but the overlap of the repertoires was low.

The low degree of peptide overlap could be due to differences in peptide fractionation by RP-HPLC. To rule out this possibility, the most abundant masses of all fractions were fragmented by MALDI-TOF/TOF-MS, obtaining a total of 73 peptide sequences: 11 from DR1, 38 from DR4, 19 from DR10, and 5 from DR15. Some of the sequences corresponded to the same peptides shared by 2 HLA–DR molecules. These peptides had the same retention time in the HPLC chromatogram. Figure 1 shows 2 examples of peptides sequenced from DR1 and DR10 from equivalent HPLC fractions. These data confirmed that the low degree of overlap was not due to differences in peptide fractionation.

Size distribution and origin of peptides bound to HLA–DR molecules. HLA–DR natural ligands were also sequenced by LC–ion-trap MS. A total of 962 unique (nonredundant) HLA–DR ligands were identified: 229 from DR1, 285 from DR4, 270 from DR10, and 178 from DR15 (see Supplementary Table 1, http://onlinelibrary.wiley.com/doi/10.1002/art.39736/abstract). The lengths of the peptides composing the 4 peptide pools were comparable. When we considered the masses obtained by LC-MALDI-TOF-MS,

Table 1. Percentage of molecular masses common to the different haplotypes detected by liquid chromatography matrix-assisted laser desorption ionization–time-of-flight mass spectrometry

	Left allele, %	Right allele, %
DR1 vs. DR4	4.35	3.88
DR1 vs. DR10	10.40	9.36
DR1 vs. DR15	6.66	3.49
DR4 vs. DR10	6.47	6.57
DR4 vs. DR15	9.00	5.30
DR10 vs. DR15	8.41	4.85

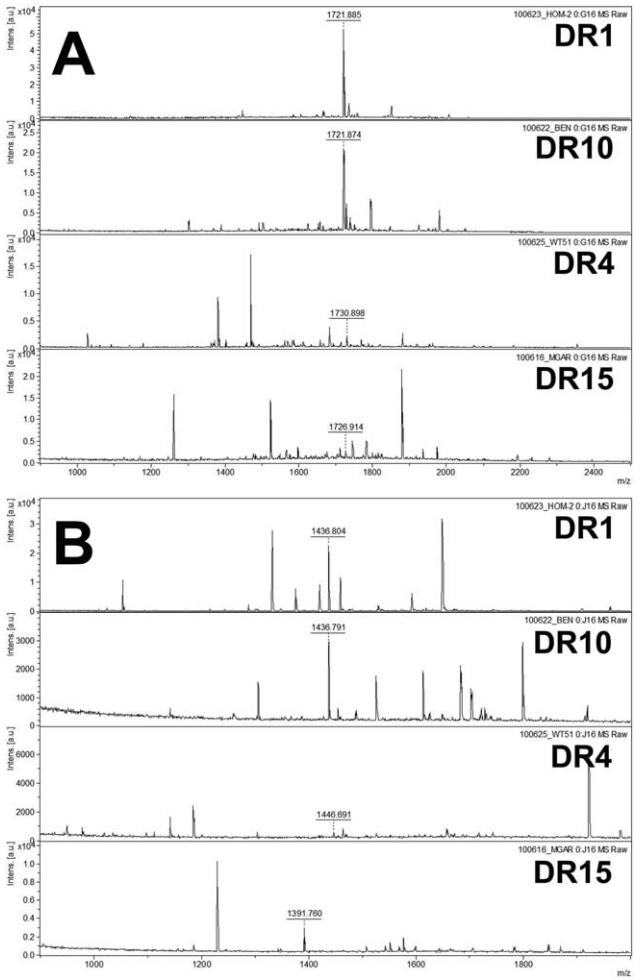

Figure 1. Two examples of the comparison of the DR1-, DR4-, DR10-, and DR15-bound peptides determined by liquid chromatography matrix-assisted laser desorption ionization–time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF-MS). Homozygous lymphoblastoid cell lines used as the source of the peptide–HLA–DR complexes were HOM-2 (DRA1*01:01/DRB1*01:01), BEN (DRA1*01:01/DRB1*10:01), WT51 (DRA1*01:01/DRB1*04:01), and MGAR (DRA1*01:01/DRB1*15:01). **A,** The spectra of fraction G16 between the different allotypes. The peak with the mass/charge (m/z) ratio of 1,721.9 corresponded to the peptide RVEYHFLSPYVSPK, as sequenced by MALDI-TOF/TOF-MS. This peptide eluted in the same fraction from DR1 and DR10 but was not detected in DR4 and DR15 in the fractions G14 to G18. The peaks with the closest m/z ratios, 1,730.9 and 1,726.9, are labeled in DR4 and DR15, respectively. **B,** The spectra of fraction J16 between the different allotypes. The peak with the m/z ratio of 1,436.8 corresponded to the peptide QQYLPLPTPKVIG, as sequenced by MALDI-TOF/TOF-MS. This peptide eluted in the same fraction from DR1 and DR10 but was not detected in DR4 and DR15 in the fractions J14 to J18. The peaks with the closest m/z ratios, 1,446.7 and 1,391.8, are labeled in DR4 and DR15, respectively. intens. = intensity.

all the peptide pools were similar, with average m/z values of 1,594.6 for DR1, 1,576.5 for DR4, 1,616.5 for DR10, and 1,645.2 for DR15 (see Supplementary Figure 2, http://onlinelibrary.wiley.com/doi/10.1002/art.39736/abstract). A similar result was obtained when we considered the peptide sequences. Thus, the average amino acid numbers were 15.3 for DR1, 15.4 for DR4, 15.3 for DR10, and 15.6 for DR15 (Figure 2A).

The cell location of the parental proteins was also analyzed. A protein was considered for each nested set. The peptide pool was derived from 121 different proteins in DR1, 79 in DR4, 128 in DR10, and 69 in DR15. The protein location described in the UniProt database (http://www.uniprot.org) and the Human Protein Reference Database (http://www.hprd.org/) was considered as previously used (30). When a protein was located in different cell compartments, the most frequent location was chosen. Most of the parental proteins of the HLA–DR ligands (ranging from 63.3% to 82.3%) were located in compartments of the endocytic pathway (membrane, secreted, vesicles, endoplasmic reticulum, and Golgi apparatus) (Figure 2B). As previously observed for these and other major histocompatibility complex (MHC) class II molecules, percentages of the peptide ligands (16.5–33.6%) were derived from cytosolic, nuclear, or mitochondrial proteins (Figure 2B).

Theoretical affinity of the HLA–DR ligandome. The theoretical binding affinity of the peptides sequenced from each of the HLA–DR molecules to this and the other allotypes was determined by calculating the IC_{50} values using a NetMHCIIpan 3.1 Server. MGAR expresses the protein product encoded in 2 loci (DRB1 and DRB5), and the alleles are known (DRB1*15:01 [also called DR2b] and DRB5*01:01 [also called DR2a]). Thus, DR2a was included in the analysis. The peptide pool associated with DR1 contained less weak binders to itself (7.86%) and to DR10 (21.83%) than to the other HLA–DR molecules (see Supplementary Table 2, http://onlinelibrary.wiley.com/doi/10.1002/art.39736/abstract). The same was seen for the DR10 peptide pool, which contained less weak binders to itself (28.89%) and to DR1 (16.30%) than to the other allotypes (Supplementary Table 2). Therefore, the peptides bound to DR1 seem to have more capacity to bind to DR10 than to the other molecules, and the same is true for the peptides bound to DR10 regarding DR1. These data are in accordance with the results observed using the LC-MALDI-TOF-MS analysis (Table 1). All the peptide pools had a lower number of theoretically weak binders for DR1 than for the other molecules. However, the peptides sequenced from DR1 had a higher preference to bind to DR10 than to DR4, DR2b, or DR2a.

Identification of common peptides between HLA–DR alleles. To directly evaluate the degree of overlap between the peptide repertoires, we analyzed the common peptides bound to the different HLA–DR molecules.

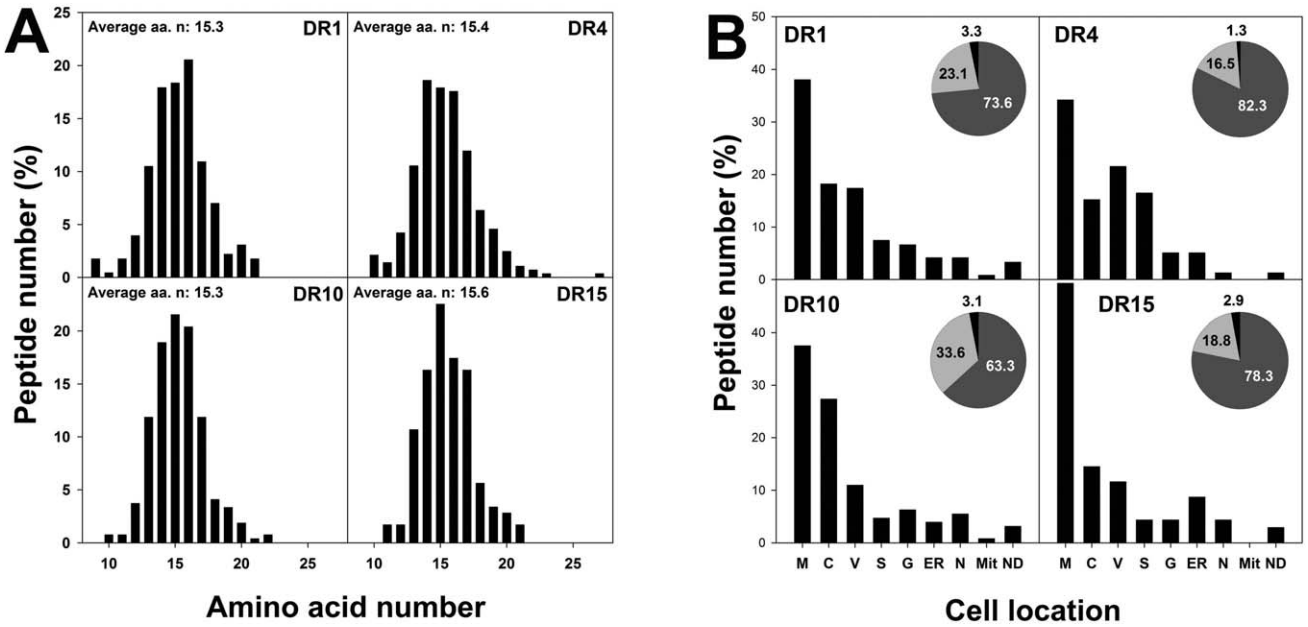

Figure 2. **A,** Peptide size distribution of the peptides sequenced from the different HLA–DR allotypes. Values are the percentage of peptides with a specific amino acid (aa) number. **B,** Major cell location of the parental proteins of the peptides sequenced from the different HLA–DR molecules. Values are the percentage of proteins for each intracellular location. Circles indicate percentages of parental proteins with a major location in the endocytic pathway (dark gray), the endogenous pathway (light gray), or not determined (black). M = membrane; C = cytosol; V = vesicular compartments; S = secreted; G = Golgi apparatus; ER = endoplasmic reticulum; N = nucleus; Mit = mitochondria; ND = not determined.

When we studied the peptides shared between HLA–DR10 and any other HLA–DR molecule, we observed that 26 peptides were common to DR10 and DR1 and 6 peptides were common to DR10 and DR4, but none were shared between DR10 and DR15. HLA–DR1 shared 1 peptide with DR4 and 6 peptides with DR15. DR4 shared 1 peptide with DR15. In addition, 2 peptides were mutually shared by DR10, DR1, and DR4, and 2 peptides were mutually shared by DR1, DR4, and DR15. Finally, 1 peptide was common to all 4 allotypes (Table 2). Therefore, DR10 shared a total of 29 peptides with DR1 (10.7% and 12.7%, respectively, of the sequenced peptides), 9 with DR4 (3.3% and 3.2%, respectively), and 1 with DR15 (0.4% and 0.6%, respectively). HLA–DR1 presented 6 common peptides with DR4 (2.6% and 2.1%, respectively) and 9 with DR15 (3.9% and 5.1%, respectively). Finally, DR4 and DR15 shared a total of 4 peptides (1.4% and 2.2%, respectively).

Some of these common peptides are part of a nested set of peptides. If we consider each peptide family as 1 unique peptide sequence, then 25 peptides were shared between the different HLA–DR molecules: 1 peptide was common to the 4 alleles; 1 peptide was common to DR1, DR4, and DR10; 18 peptides were common to DR1 and DR10; 2 peptides were common to DR10 and DR4; 2 peptides were common to DR1 and DR15; and 1 peptide was common to DR4 and DR15. Therefore, these data clearly confirm that the peptide repertoire associated with HLA–DR1 is the most similar to that bound to DR10.

Features of the common peptides between HLA-DR molecules. To evaluate similar features of the common peptides, we considered the only peptide set with a sufficiently high number of components: the peptides shared between DR10 and DR1. As mentioned above, 18 of the 25 shared nested sets were common to DR1 and DR10 alone. There was a clear bias toward Leu at the P4 position of the anchor core among these peptides (Table 3). Fourteen of the 18 common peptides (77.8%) contained Leu^4, while only 2 of the 7 remaining shared peptides (28.6%) contained Leu^4. These peptides contained Ala, Glu, Asp, Ile, and Leu (Table 3). The peptide SPNIVIALAGNKAD presented VIALAGNKA as the theoretical core for DR1, DR4, DR10, and DR2b, while IVIALAGNK was the theoretical core for DR2a. Approximately 35% of the ligands eluted from DR1 and 38% of those eluted from DR10 contained Leu^4. However, only 4% and 18% of DR4 and DR15 ligands, respectively, contained Leu^4. Thus, although the presence of Leu^4 is accepted in all allotypes, it is clearly favored in DR1 and

Table 2. Common peptides associated with different HLA–DR molecules*

Peptide sequence	Protein	HOM-2 (DR1)	BEN (DR10)	WT51 (DR4)	MGAR (DR15)
LPSYEEALSLPSKTPE	Lysosomal-associated transmembrane protein 5	X	X	X	X
VTQEIVTERSVSSRQA	Desmoglein 2	X	X	X	
VVLPSYEEALSLPSKTPE	Lysosomal-associated transmembrane protein 5	X	X	X	
LPSYEEALSLPSKTP	Lysosomal-associated transmembrane protein 5	X		X	X
LPSYEEALSLPSKTPEG	Lysosomal-associated transmembrane protein 5	X		X	X
AEPNYHSLPSARTDEQ	Regulator complex protein LAMTOR1	X	X		
APSTYAHLSPAKTPPP	Lipolysis-stimulated lipoprotein receptor	X	X		
APSTYAHLSPAKTPPPP	Lipolysis-stimulated lipoprotein receptor	X	X		
APSTYAHLSPAKTPPPPA	Lipolysis-stimulated lipoprotein receptor	X	X		
ASAFAFLAPARAIL	Solute carrier family 23 member 2	X	X		
DARSYTAAVANRAKGGG	Myotubularin-related protein 4	X	X		
DFYKILGVPRSASIK	DnaJ homolog subfamily B member 11	X	X		
IEKFEKEAAEMGKG	Elongation factor 1α1	X	X		
ISQELEELRAEQQRL	Low-affinity immunoglobulin epsilon Fc receptor	X	X		
KGRLDYLSSLKVKGLV	4F2 cell-surface antigen heavy chain	X	X		
LPTSFGSLAPAPLVFPGP	Ubiquitin protein ligase E3 RNF167	X	X		
LPVILDIIKGEMSRPG	Proactivator polypeptide	X	X		
NPKTFFILHDINSDG	Nucleobindin 1	X	X		
QQYLPLPTPKVIG	70-kd heat-shock protein 13	X	X		
QQYLPLPTPKVIGID	70-kd heat-shock protein 13	X	X		
QYLPLPTPKVIGID	70-kd heat-shock protein 13	X	X		
RVEYHFLSPYVSP	Transferrin receptor protein 1	X	X		
RVEYHFLSPYVSPK	Transferrin receptor protein 1	X	X		
RVEYHFLSPYVSPKE	Transferrin receptor protein 1	X	X		
SPEDEYELLMPHRIS	Germinal center–associated signaling and motility protein	X	X		
SPEDEYELLMPHRISS	Germinal center–associated signaling and motility protein	X	X		
SPERPFLAILGGAKVADK	Phosphoglycerate kinase 1	X	X		
SPTNYHFLSSPKEA	Beta-mannosidase	X	X		
TPDPSKFFSQLSSEHGGD	Interleukin-2 receptor subunit beta	X	X		
TPDPSKFFSQLSSEHGGDV	Interleukin-2 receptor subunit beta	X	X		
VDKVIQAQTAFSANPA	Syntenin 1	X	X		
DDTQFVRFDSDAASPR	HLA class I histocompatibility antigen, B-14 α-chain		X	X	
VDDTQFVRFDSDAASPR	HLA class I histocompatibility antigen, B-14 α-chain		X	X	
GNYRIESVLSSSG	Ganglioside GM2 activator		X	X	
TGNYRIESVLSSSG	Ganglioside GM2 activator		X	X	
TGNYRIESVLSSSGK	Ganglioside GM2 activator		X	X	
TTGNYRIESVLSSSGK	Ganglioside GM2 activator		X	X	
VTQEIVTERSVSSRQAQ	Desmoglein2	X		X	
EDPAFYMLKGKLQYE	Interleukin-21 receptor	X			X
PAFYMLKGKLQYE	Interleukin-21 receptor	X			X
SPNIVIALAGNKAD	Ras-related protein Rab-5C	X			X
SPNIVIALAGNKADL	Ras-related protein Rab-5C	X			X
SPSIVIALAGNKAD	Ras-related protein Rab-5B	X			X
SPSIVIALAGNKADL	Ras-related protein Rab-5B	X			X
DHNFVKAINAIQKS	Dipeptidylpeptidase 1			X	X

* Homozygous lymphoblastoid cell lines used as the source of the peptide–HLA–DR complexes were HOM-2 (DRA1*01:01/DRB1*01:01), BEN (DRA1*01:01/DRB1*10:01), WT51 (DRA1*01:01/DRB1*04:01), and MGAR (DRA1*01:01/DRB1*15:01). An X to the right of a peptide sequence and below an HLA–DR molecule indicates that peptide sequence is associated with that HLA–DR molecule.

DR10. On the other hand, most of the common peptides that included DR4 as one of the molecules from which they were eluted contained an acidic amino acid in the P4 core position. Approximately 29% of peptides eluted from DR4 had Asp or Glu at P4, in comparison with 22% of peptides eluted from DR1, 15% of peptides eluted from DR10, and 12% of peptides eluted from DR15.

Peptides shared by DR1 and DR10 had a preference for basic residues at the P8 core position. Thus, 61.2% of the common peptides had a basic residue at this position (27.8% Arg, 27.8% Lys, and 5.6% His). None of the peptides shared with other molecules had basic residues at this position. However, all the molecules accepted basic residues at P8 (33.6% for DR1, 19.8% for DR4, 31.0% for DR10, and 24.0% for DR15).

Binding assay of shared peptides to HLA–DR molecules. The peptides VTQEIVTERSVSSRQA, SPSIVIALAGNKADL, DHNFVKAINAIQKS, and APSTYAHLSPAKTPPP were synthesized, and their binding affinity to DRB1*01:01, DRB1*04:01, DRB1*10:01,

Table 3. Theoretical cores of the common peptides associated with HLA–DR molecules and P4 residues used by them*

Peptide binding core†	HOM-2 (DR1)	BEN (DR10)	WT51 (DR4)	MGAR (DR15)	P4 residue
YEE**A**LSLPS	X	X	X	X	A
IVT**E**RSVSS	X	X	X		E
YHS**L**PSART	X	X			L
YAH**L**SPAKT	X	X			L
FAF**L**APARA	X	X			L
YTA**A**VANRA	X	X			A
YKI**L**GVPRS	X	X			L
FEK**E**AAEMG	X	X			E
LEE**L**RAEQQ	X	X			L
LDY**L**SSLKV	X	X			L
FGS**L**APAPL	X	X			L
LDI**I**KGEMS	X	X			I
FFI**L**HDINS	X	X			L
YLP**L**PTPKV	X	X			L
YHF**L**SPYVS	X	X			L
YEL**L**MPHRI	X	X			L
LAI**L**GGAKV or FLAI**L**GGAK‡	X	X			L or I
YHF**L**SSPKE	X	X			L
FSQ**L**SSEHG	X	X			L
IQA**Q**TAFSA	X	X			Q
VRF**D**SDAAS		X	X		D
YRI**E**SVLSS		X	X		E
FYM**L**KGKLQ	X			X	L
VIA**L**AGNKA or IVI**A**LAGNK§	X			X	L or A
FVKA**I**NAIQ or VKA**I**NAIQK¶			X	X	A or I

* Homozygous lymphoblastoid cell lines used as the source of the peptide–HLA–DR complexes were HOM-2 (DRA1*01:01/DRB1*01:01), BEN (DRA1*01:01/DRB1*10:01), WT51 (DRA1*01:01/DRB1*04:01), and MGAR (DRA1*01:01/DRB1*15:01). An X to the right of a peptide binding core and below an HLA–DR molecule indicates that peptide binding core is associated with that HLA–DR molecule.
† Cores were selected using a NetMHCIIpan 3.1 Server. P4 residues are shown in boldface.
‡ FLAILGGAK to DRB1*10:01.
§ IVIALAGNK to DRB5*01:01.
¶ VKAINAIQK to DRB5*01:01.

DRB1*15:01, and DRB5*01:01 was experimentally calculated with a binding assay. These peptides were differentially found in the repertoires of these allotypes: VTQEIVTERSVSSRQA was sequenced from DR1, DR4, and DR10; APSTYAHLSPAKTPPP from DR1 and DR10; SPSIVIALAGNKADL from DR1 and DR15; and DHNFVKAINAIQKS from DR4 and DR15. All the peptides had high affinity for the HLA–DR molecule from which they were identified (Table 4). In addition, the peptides had low affinity to the alleles from which they were not sequenced.

A few peptides not detected in a specific peptide repertoire can bind to the corresponding molecule with a reasonably good affinity. Thus, the peptide VTQEIVTERSVSSRQA was observed to bind to DRB1*15:01 with high affinity (IC_{50} = 0.55 n*M*); the peptide DHNFVKAINAIQKS bound to DRB1*10:01 with an IC_{50} of 1.75 n*M*, although this value was 25 times lower than its binding

Table 4. Experimental binding affinity of shared peptides associated with HLA–DR molecules*

Peptide bound to	Peptide sequence†	DRB1*01:01	DRB1*04:01	DRB1*10:01	DRB1*15:01	DRB5*01:01
DR1–DR4–DR10	VTQE**IVTERSVSS**RQA	8.26	0.33	3.36	0.55	>50
DR1–DR10	APS**TYAHLSPAKT**PPP	0.15	>50	0.09	>50	18.15
DR1–DR15	**SPSIVIALAGNKA**DL‡	0.19	>50	>50	>50	<0.05
DR4–DR15	DHN**FVKAINAIQ**KS	>50	0.04	1.75	<0.05	0.07
–	Controls	0.07	0.16	0.93	0.05	0.1

* Values are 50% inhibition concentrations.
† The theoretical binding core is highlighted in boldface.
‡ The theoretical core for DRB5*01:01 is IVIALAGNK as defined by a NetMHCIIpan 3.1 Server.

to the molecules where it was identified (0.04 n*M* to DRB1*04:01, <0.05 n*M* to DRB1*15:01, and 0.07 n*M* to DRB5*01:01); and the peptide APSTYAHLSPAKTPPP bound to DRB5*01:01 with a much lower affinity than that to the alleles from which the peptide was sequenced (18.15 n*M* to DRB5*01:01 versus 0.15 n*M* to DRB1*01:01 and 0.09 n*M* to DRB1*10:01). Therefore, the experimental binding results were highly consistent with the data of peptide sequencing and the source from which they were sequenced.

Modeling of the peptide–MHC interaction. The peptide with sequence VTQEIVTERSVSSRQA, derived from desmoglein 2, was found to be bound to the 3 HLA–DR molecules associated with RA but not to DR15. The binding assays confirmed the high affinity of this peptide for DR1, DR4, and DR10 and indicated that it could also bind to DR2b but not to DR2a (Table 4). The interaction of the peptide core (IVTERSVSS) with the 4 HLA–DRB1 molecules and HLA–DRB5*01:01 was studied by computational modeling. The results were compatible with the binding data, predicting a lowest binding affinity of the peptide for DR2a. The models and computed relative binding scores are shown in Supplementary Figure 3, http://onlinelibrary.wiley.com/doi/10.1002/art.39736/abstract.

DISCUSSION

Herein we present a comparison of the HLA–DR peptide repertoires of 3 of the most strongly RA-associated HLA–DR allotypes containing the SE (DRB1*01:01, DRB1*04:01, and DRB1*10:01) and a non–RA-associated HLA–DR molecule (DRB1*15:01) in order to analyze the degree of overlap of these repertoires and deduce structural features of the peptides shared by these allotypes. Different studies have analyzed the peptide pools of several of these HLA–DR molecules (19,21,35–37), but not in a comparative manner. The identification of structural features of the peptides bound to differentially RA-associated alleles is required to define the putative target of the T cell immune response generated by RA-related molecules. These peptides might be used for the development of cell therapies (e.g., generation of tolerogenic dendritic cells, development of peptide vaccines).

The promiscuity of MHC class II molecules regarding the peptides they can bind was demonstrated long ago (21,22). In the case of RA, 2 of the SE molecules, DR1 and DR4, bind an immunodominant type II collagen peptide that interacts with the same residues of both molecules (38). However, the degree of overlap of the peptide repertoires bound to HLA–DR alleles differentially associated with RA had not yet been measured.

To do this, we analyzed the peptide pools bound to these 4 HLA–DR molecules. First, using LC-MALDI-TOF/TOF-MS, we collected and analyzed 240 fractions of each peptide pool. Although the peptide repertoires contained common peptides, the global overlap between the peptide pools bound to 2 HLA–DR molecules was low, ~10% in the 2 more similar repertoires (DR1 and DR10). Analysis of the peptides sequenced by MS clearly confirmed this low degree of overlap and also confirmed that DR1 and DR10 had the most similar peptide repertoires, sharing 12.7% and 10.7%, respectively, of peptides. The percentage of peptides common to any other pair of molecules did not exceed 5.1%. Using similar technology, the overlap of the peptide pools described previously for several HLA–B27 subtypes was much higher (23,24). Several subtypes of HLA–DR4 are clearly associated with RA (DRB1*04:01, DRB1*04:04, DRB1*04:05, and DRB1*04:08), while others can be considered low-risk alleles (DRB1*04:02) or non–RA-associated alleles (DRB1*04:03 and DRB1*04:07) (5). The anchor motifs of HLA–DRB1*04:01, HLA–DRB1*04:02, and HLA–DRB1*04:04 have been identified based on peptide ligands (37), although the overlap of the repertoires was not described.

One peptide derived from desmoglein 2, VTQEIVTERSVSSRQA, was sequenced from DR1, DR4, and DR10 peptide pools. The peptide core–MHC modeling of the putative cores correlated very well with the empirical binding data. Thus, this peptide binds with high affinity to DR1, DR4, DR10, and DR2b, but not to DR2a. Some promiscuous peptides have been analyzed in the literature, but DR10 had not yet been included as one of the HLA–DR alleles. This peptide contains Glu^4 as one of the anchor residues.

Another peptide, LPSYEEALSLPSKTPE, derived from lysosomal-associated transmembrane protein 5, was sequenced from all the allotypes analyzed. Although this self-peptide would not have any implications for immunity, to our knowledge it is the first natural ligand sequenced from 4 different RA-associated HLA–DR molecules, with the exception of CLIP. This finding confirmed the existence of peptides that are very promiscuous regarding the HLA–DR molecules to which they can bind. However, the fact that only 1 such peptide was found indicates that the number of peptides with the capacity to bind to many HLA–DR alleles is limited.

Regarding the theoretical affinity data, DR1 was the allele to which more peptides could bind with high affinity. These data suggest that a NetMHCIIpan 3.1 Server overestimates the binding affinity with DR1 in comparison with other allotypes. This was confirmed with the binding affinity data. Although the theoretical data correlated well with most of the peptides and alleles, some discrepancies

were detected. In all cases, the peptides were good binders for DR1, including the peptide DHNFVKAINAIQKS, which was not detected as a DR1 ligand and was not bound to DR1 in the binding assay.

The identification of the binding core was made using a NetMHCIIpan 3.1 Server. Structural analysis is the only way to conclusively identify the peptide-binding core. However, although we recognize that a few cores assigned can be wrong, this number should be very low and should not affect the global analysis.

Only the number of peptides shared between DR1 and DR10 allowed the search for some structural features of the common peptides. Two features were observed: most of the peptides had Leu in P4 and basic residues in P8. These residues were enriched when compared with their presence in the individual peptide repertoires, as the abundance of Leu^4 and basic residues at P8 in the shared peptides was approximately double that in DR1 or DR10 alone. Therefore, although a higher number of peptides is required, these data suggest that the peptides containing Leu^4 and basic residues at P8 are favored to bind to DR1 and DR10 and not to other molecules. One exception was the peptide SPSIVIALAGNKADL, which was found in the peptide repertoires of DR1 and DR15. The binding assay demonstrated that this peptide was good at binding to DR1 and DR2a, but not to DR4, DR10, and DR2b. The core for DR2a was IVIALAGNK, which lacks Leu^4 and Lys^8.

The polar residues Ser and Asn are the most abundant residues in the P9 core position of DR10 (19% and 20%, respectively, of the peptide families). Ser was also detected in the other peptide pools (11% in DR1, 21% in DR4, and 10% in DR15). However, Asn was not detected in any of the DR1 and DR15 ligands, and only 2% of the peptides bound to DR4 had Asn^9. Thus, although the P9 core position accepted a high number of different residues, mostly polar and small aliphatic residues, only DR10 had Asn as a favored residue. Therefore, our data strongly suggest that peptides shared by DR1 and DR10 are enriched in Leu^4 and basic residues at P8 and do not contain Asn at P9.

Approximately 60% of RA patients have citrullinated protein–specific antibodies (39), which correlate highly with expression of the SE allotypes (40). The identification of citrullinated peptides directly from the eluted peptide pool is very difficult (41). Glutamine is an amino acid with physical and chemical features similar to those of citrulline. The presence of Gln^4 may be an indirect clue to the acceptance of citrulline at the P4 core position. The 4 peptide repertoires contained peptides with Gln^4 (6.4% for DR1, 14.3% for DR4, 6.7% for DR10, and 5.6% for DR15). Thus, the analysis of these peptide pools suggests that DR4 may be the allele with the higher probability of presenting peptides containing citrulline in the P4 core position. The binding of peptides with this modification to DR4 has been described (42). DR15 had fewer peptides with Gln^4, but the percentage was very similar to those for DR1 and DR10.

The characterization of peptide sequence patterns between common peptides bound to RA-associated molecules and absent from non–RA-associated allotypes may be used to identify new T cell epitopes derived from autoantigens in joints and differentially presented in RA patients.

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All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Álvarez had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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