Title:

A comparative analysis of the peptide repertoires of HLA-DR molecules differentially associated to rheumatoid arthritis

Subtitle: HLA-DR peptidomes in rheumatoid arthritis

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Keywords

Rheumatoid arthritis, HLA-DR, shared epitope, mass spectrometry, peptidome.

Abbreviations

RA, rheumatoid arthritis; **HLA**, human leukocyte antigen; **SE**, shared epitope; **TCR**, T cell receptor; **LCLs**, lymphoblastoid cell lines; **pHLA-DR**, peptide-HLA-DR complexes; **MS**, mass spectrometry; **PBS**, phosphate buffered saline; **TFA**, trifluoroacetic acid; **RP-HPLC**, reverse phase-high performance liquid chromatography; **MALDI-TOF**, matrix-assisted laser desorption/ionization-time of flight; **FDR**, False Discovery Rate; **MHC-II**, class II major histocompatibility complex molecules.

Abstract

OBJECTIVE: Our aims were to evaluate the similarity of the peptide repertoires bound to HLA-DR molecules that are differentially associated with rheumatoid arthritis and 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 (associated) and HLA-DRB1*15:01 (non-associated) were purified and analyzed by LC-MALDI and LC-ion-Trap mass spectrometry. The peptide pools from each allotype were compared in terms of size, protein origin, composition and affinity (theoretical and experimental with some peptides). Finally, one peptide sequenced from DR1, DR4 and DR10, but not from DR15 was modeled in complex with all four HLA-DRB1 molecules and HLA-DRB5*01:01.

RESULTS: A total of 6309 masses and 962 unique peptide sequences were compared. DR10 shared 29 peptides with DR1, 9 with DR4 and 1 with DR15; DR1 shared 6 common 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 are the most similar molecules regarding the peptides that they can share. The common peptides between these molecules contain a high proportion of Leu at P4 and basic residues at P8 binding core positions.

CONCLUSION: The degree of overlap between the peptide repertoires associated to different HLA-DR molecules is low. The repertoires associated to DR1 and DR10 have the highest similarity among the molecules analyzed (around 10% overlap). Among the shared peptides between DR1 and DR10 a high proportion contained Leu4 and basic residues at P8 position of the binding core.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease affecting peripheral joints. The etiopathogenesis of the disease is complex involving both environmental and genetic factors. The strongest genetic association is with the human leukocyte antigen (*HLA*)-*DRB1* locus (1-3), although other genes have also been related with 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, as *HLA-DRB1**03:01, *HLA-DRB1**07:01, *HLA-DRB1**08:01 or are considered as non-associated or low-risk alleles, as *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 the T cell recognition, by either directly interacting with the T cell receptor (TCR) or defining the HLA-DR-associated peptide repertoire by direct interaction with the peptides. One of the molecular contacts between the HLA-DR molecule and the peptide is among the residue 71 of the DR β 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 in the serum of

RA patients of citrullinated protein-specific antibodies is closely linked to the expression of the SE alleles (10, 11).

HLA-DR4 is associated to 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 described by our group by sequencing its peptide repertoire partially (19). The anchor motif was subsequently confirmed by other techniques with small differences (20). Theoretically, there should be a higher degree of overlap between the peptide repertoires bound by SE-containing HLA-DR allotypes, such as DRB1*01:01 and DRB1*04:01, than with the repertoires bound to other low-risk or negatively associated molecules, as DRB1*15:01 or DRB1*07:01 (19). Such repertoires could be inferred based on bioinformatics based prediction tools. However, the available tools use different algorithms and differ in their assignments of the 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-non-related molecules.

HLA-DR molecules are promiscuous regarding the peptides they can bind (21, 22). However, a direct quantification of the overlapping degree between different HLA-DR allotypes differentially associated to RA has not yet been performed. This approach has been successfully performed to compare the peptide pools sequenced from different HLA-B27 subtypes and to quantify the overlapping degree of these peptide repertoires (23-25). Here, we describe a comparison of the peptide repertoires bound to HLA-DR alleles differentially associated to RA. Our analysis included three of the most clearly

RA-associated alleles: *HLA-DRB1*01:01*, *HLA-DRB1*04:01*, *HLA-DRB1*10:01*, and a non-associated allele, *HLA-DRB1*15:01*. Homozygous lymphoblastoid cells (LCSs) were used as the source of the peptide-HLA-DR complexes (pHLA-DR), which were purified by immunoaffinity chromatography. After acid elution and ultrafiltration, peptides were analyzed by LC-MALDI AND LC-ion Trap mass spectrometry (MS). The analysis of the peptide pools indicated that the overlapping of the peptide repertoires of different HLA-DR molecules is relatively low. The two most similar HLA-DR peptide repertoires are those from DR1 and DR10 which shared a high proportion of peptides with Leu at the P4 binding core position and basic residues at P8.

Materials and methods

Cell lines and antibody

Homozygous EBV-transformed B-lymphoblastoid cell lines (B-LCL) from the 10th International Histocompatibility Workshop include 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-LCL BEN (*DRA1*01:01/DRB1*10:01*) was previously described (19). The sequence alignment of the *DRB1* alleles is shown in the Supplementary figure 1. Cells were grown in roller bottles in RPMI (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Cells were washed three times in cold phosphate buffered saline (PBS), and dry pellets stored at -70°C until processing. The HLA-DR-specific monoclonal antibody B8.11.2 (IgG2b) (26) was used for pHLA-DR purification.

HLA-DR-associated peptide pool purification

Peptide-HLA-DR complexes were purified as described (19, 27). Procedures are detailed in Supporting information.

LC-MALDI-TOF/TOF

Peptides were fragmented by reverse phase high performance liquid chromatography (RP-HPLC). Fractions were taken every 15 seconds and analyzed in a matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) UltrafleXtreme (Bruker daltonics, Bremen, Germany).

The peptide repertoires from the different allotypes were compared by pairs using the software MSHandler (28), setting the system as follows: the selected mass range was 1100-3000 Da; tolerances used to judge two mass signals as equivalent peptide were set

as ± 0.25 Da/z for the m/z value and ± 2 elution positions for the chromatographic retention time; the signal intensity cut off for the whole spectrum was 100, in absolute value of intensity units, and the specific signal cut off, discarding signals likely assigned as specific in a mistaken way by lack of instrumental accurateness, was 500; the specific signals search width, discarding signals that are 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 two different chromatographies show two equivalent masses corresponding to different peptides, was specifically calculated for each comparative analysis performed. FDR calculation was carried out by determining the overlay 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 which were distributed along 250 theoretical chromatographic fractions, placing 20-30 masses in each fraction.

Ion Trap Mass Spectrometry and sequence identification

Peptides were analyzed by a tridimensional ion trap mass spectrometer (Esquire HCT Ultra, Bruker, Bremen, Germany) (19, 29) and a high-resolution mass spectrometer, linear trap quadrupole (LTQ-Orbitrap XL, ThermoFisher, San Jose, CA) (29-31). Procedures are detailed in Supporting information.

Predicted binding affinity

The theoretical binding of each peptide to the different HLA-DR molecules was calculated using the NetMHCIIpan 3.1 Server. The IC₅₀ value was considered. Peptides

with an IC₅₀ lower than 50 nM were defined as strong binders (SB); between 50 and 500 nM as intermediate binders (IB); and higher than 500 nM as weak binders (WB).

Peptide HLA-DR binding assay

The experimental affinity of some peptides to HLA-DR molecules was assayed as previously described (30). 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), DR2a (DRA1*01:01/DRB5*01:01) proteins were used for peptide binding assays. Increasing concentrations of each non-biotinylated test peptide were incubated in competition with 0.02 mM biotinylated haemagglutinin HA₃₀₆₋₃₁₈ peptide in wells coated with HLA-DR1 or HLA-DR4 protein, 0.01 mM biotinylated TT560–571 peptide in wells coated with HLA-DR10 protein, and 0.01 mM biotinylated modified GAD555-567 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. After washing, residual biotinylated reference peptide was labeled using europium conjugated streptavidin (Perkin Elmer) and quantified using a Victor3 D time resolved fluorometer (Perkin Elmer). Peptide binding curves were simulated by non-linear regression with Prism software (Version 4.03, GraphPad Software Inc.) using a sigmoidal dose-response curve. IC₅₀ 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 (32) (see Supporting Information). 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

Overlapping of the HLA-DR peptide repertoires

The HLA-DR peptide pools that were eluted from DR1, DR4, DR10 and DR15 respectively were purified and analyzed by two different techniques: LC-MALDI-TOF/TOF 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. A total of 6309 signals with m/z ranging from 1100 to 3000 were considered: 1594 from DR1, 1278 from DR4, 1248 from DR10 and 2189 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% of the masses analyzed, respectively (Table 1). These data confirmed the promiscuity of the peptides bound to HLA-DR molecules, but the overlapping of the repertoires was low.

The low peptide overlapping degree could be due to differences in the peptide fractionation by RP-HPLC. To discard it, the most abundant masses of all fractions were fragmented by MALDI-TOF/TOF, 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 two HLA-DR molecules. These peptides had the same retention time in the HPLC chromatogram. The figure 1 shows two examples of peptides sequenced from DR1 and DR10 from equivalent HPLC fractions. These data confirmed that the low overlapping degree were not due to differences in the peptide fractionation.

Size distribution and origin of the peptides bound to HLA-DR molecules

HLA-DR natural ligands were also sequenced by LC-ion Trap MS. A total of 962 unique (non-redundant) HLA-DR ligands were identified: 229 from DR1, 285 from DR4, 270 from DR10 and 178 from DR15 (Supplementary table 1). The lengths of the peptides composing the four peptide pools were comparable. When we consider the masses obtained by LC-MALDI, all the peptide pools were similar, with an average m/z of 1594.6 for DR1, 1576.5 for DR4, 1616.5 for DR10 and 1645.2 for DR15 (Supplementary figure 2). A similar result was obtained considering the peptide sequences. Thus, the average amino acid number was 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 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 (29). When a protein was located in different cell compartments, the most usual 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 MHC class II molecules (MHC-II), a percentage of the peptide ligands derived from cytosolic, nuclear or mitochondrial proteins, ranging from 16.5% to 33.6% (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 IC50 values using the NetMHCIIpan-3.1 Server (33). MGAR expresses the protein product encoded

in two *loci* (*DRB1* and *DRB5*) and the alleles are known (*DRB1*15:01*, also called DR2b and *DRB5*01:01* or DR2a). Thus, DR2a was included in the analysis. The peptide pool associated to DR1 contained less weak binders to itself (7.86%) and to DR10 (21.83%) than to the other HLA-DR molecules (Supplementary table 2). 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 occurs for the peptides bound to DR10 regarding DR1. This data are in accordance with the results observed by the LC-MALDI-TOF analysis (Table 1). All the peptide pools have a lower number of theoretically weak binders for DR1 than for the other molecules. However, the peptides sequenced from DR1 have 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. 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; 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 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, one peptide was common to all four allotypes (Table 2). Therefore, DR10 shared a total of 29 peptides with DR1 (10.7% and 12.4% of the sequenced peptides, respectively), 9 with DR4 (3.3% and 3.2%) and 1 with DR15 (0.4% and 0.6%). HLA-DR1 presented 6 common peptides with DR4 (2.6% and 2.1%) and 9

with DR15 (3.9% and 5.1%). Finally, DR4 and DR15 shared a total of 4 peptides (1.4% and 2.2%).

Some of these common peptides are part of a nested set of peptides. If we consider each peptide family as one unique peptide sequence, then 25 peptides were shared between the different HLA-DR molecules: 1 peptide was common to the 4 alleles; 1 peptide to DR1, DR4 and DR10; 18 peptides to DR1 and DR10; 2 peptides to DR10 and DR4; 2 peptides to DR1 and DR15; and 1 peptide to DR4 and DR15. Therefore, these data clearly confirm that the peptide repertoire associated to 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 number of components high enough: the peptides shared between DR10 and DR1. As said above, 18 of the 25 shared nested sets were common to DR1 and DR10. There was a clear bias to Leu at the P4 position of the anchor core among these peptides (Table 3). Fourteen of the 18 common peptides (77.8%) contained Leu4, while only 2 of the 7 remaining shared peptides (28.6%) comprised Leu4. These peptides contained Ala, Glu, Asp 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. About 35% of the ligands eluted from DR1 and 38% from DR10 contained Leu4. However, only 4% and 18% of the DR4 and DR15 ligands presented Leu4. Thus, although the presence of Leu4 is accepted in all allotypes, it is clearly favored in DR1 and 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. About 29% of the

peptides eluted from DR4 had Asp or Glu at P4, in comparison with the 22% from DR1, 15% from DR10 and 12% 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 (DR1: 33.6%, DR4: 19.8%, DR10: 31.0% and DR15: 24.0%).

Binding assay of shared peptides to HLA-DR molecules

The peptides VTQEIVTERSVSSRQA, SPSIVIALAGNKADL, DHNFVKAINAIQKS and APSTY AHLSPAKTPPP were synthesized and their binding affinity to DRB1*01:01, DRB1*04:01, DRB1*10:01, 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; APSTY AHLSPAKTPPP 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 a high affinity ($IC_{50} = 0.55$ nM); the peptide DHNFVKAINAIQKS to DRB1*10:01 with a $IC_{50} = 1.75$ nM, although this was 25 times lower than to the molecules where it was identified (0.04 nM to DRB1*04:01 and <0.05 nM to DRB1*15:01 or 0.07 nM to DRB5*01:01); and the peptide APSTY AHLSPAKTPPP to DRB5*01:01, to which the

affinity was also much lower than to the alleles from which the peptide was sequenced (18.15 nM to DRB5*01:01 vs. 0.15 nM to DRB1*01:01 and 0.09 nM 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 three HLA-DR molecules associated to 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 four HLA-DRB1 molecules and HLA-DRB5*01:01 was studied by computational modeling. The results are 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.

Discussion

Here, we present a comparison of the HLA-DR peptide repertoires of three 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-associated HLA-DR molecule (DRB1*15:01) in order to analyze the overlapping degree 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, 34-36), but not as a comparative study. The identification of structural features of the peptides bound to differentially RA-associated alleles is required to define putative target of the T cell immune response presented by RA-related molecules. These peptides would have the potential to be used for the development of cell therapies (as the generation of tolerogenic dendritic cells, development of peptide vaccines, etc.).

The promiscuity of the MHC-II molecules to bind identical peptides was demonstrated long-time ago (21, 22). In the case of RA, two of the SE molecules, DR1 and DR4, bind an immunodominant type II collagen peptide that interacts with the same residues of both molecules (37). However, the overlapping degree of the peptide repertoires bound to HLA-DR alleles differentially associated to RA has not yet been measured. For that, we have analyzed the peptide pools bound to these four HLA-DR molecules. First, we compared by LC-MALDI-TOF/TOF 240 fractions of each peptide pool. Although the peptide repertoires contained common peptides, the global overlap between the peptide pools bound to two HLA-DR was low, around 10% in the two more similar repertoires (DR1 and DR10). Analysis of the peptides sequenced by mass spectrometry clearly confirmed this low overlapping degree and that DR1 and DR10 had the most similar peptide repertoires, sharing 12.7% and 10.7% of the peptides, respectively. The percentage of common peptides by any other pair of molecules did not exceed 5.1%.

The overlap of the peptide pools described previously for several HLA-B27 subtypes using similar technology was much higher (23, 24). Several subtypes of HLA-DR4 are clearly associated to RA (*DRB1*04:01*, *DRB1*04:04*, *DRB1*04:05*, *DRB1*04:08*), while others can be considered as low risk alleles (*DRB1*04:02*) or non-associated alleles (*DRB1*04:03*, *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 (36), although the overlapping 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 empiric binding data. Thus, this peptide binds with high affinity to DR1, DR4, DR10 and DR2b, but not to DR2a. In the literature, some promiscuous peptides have been analyzed, but DR10 had not been yet included as one of the HLA-DR alleles. This peptide contains Glu4 as one of the anchor residues.

Other peptide, LPSYEEALSLPSKTPE, derived from Lysosomal-associated transmembrane protein 5, was sequenced from all the allotypes analyzed. Although this self-peptide would not have any immune implications, is in our knowledge the first natural ligand sequenced from four differently RA-associated HLA-DR molecules with the exception of CLIP. This finding confirmed the existence of peptides very promiscuous regarding to the HLA-DR molecules they can bind to. However, the fact that only one 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 the NetMHCIIpan3.1 software overestimate the binding affinity with DR1 in comparison with other allotypes. This was confirmed with the data of the binding affinity. 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, included 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 the NetMHCIIpan 3.1 software. The only way to be conclusive in the identification of the peptide binding core is with structural analysis. However, although we do not discard that a few cores assigned can be wrong, this number should be very low and should not affect to the global analysis.

Only the number of peptides shared between DR1 and DR10 allowed the search of 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 Leu4 and basic residues at P8 in the shared peptides is around the double than in DR1 or DR10 alone. Therefore, although a higher number of peptides is required, these data suggest that the peptides containing Leu4 and basic residues at P8 are favored to bind to DR1 and DR10 and not to other molecules. One exception is the peptide SPSIVIALAGNKADL which was found in the peptide repertoires of DR1 and DR15. The binding assay demonstrated that this peptide was a good binder for DR1 and DR2a, but not to DR4, DR10 and DR2b. The core for DR2a was IVIALAGNK, lacking the Leu4 and Lys8.

The polar residues Ser and Asn are the most abundant residues in the P9 core position of DR10 (19% and 20% of the peptide families, respectively). 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 Asn9. Thus, although the P9 core position accepted a high number of

different residues, mostly polar and small aliphatic residues, only DR10 have Asn as a favoured residue. Therefore, our data strongly suggest that peptides shared by DR1 and DR10 are enriched in Leu4, basic residues at P8 and do not contain Asn at P9.

About 60% of RA patients contain antibodies anti-citrullinated proteins (38) what highly correlates with the expression of the SE allotypes (39). The identification of citrullinated peptides directly from the eluted peptide pool is very difficult (40). Glutamine is an amino acid with similar physicochemical features to citrulline. The presence of Gln4 may be an indirect clue of the acceptance of citrulline at the P4 core position. The four peptide repertoires contained peptides with Gln4 (DR1: 6.4%, DR4: 14.3%, DR10: 6.7 and DR15: 5.6%). Thus, the analysis of these peptide pools suggests that DR4 may be the allele with the higher probability to present peptides containing citrulline in the P4 core position. The binding of peptides with this modification to DR4 have been described (41). DR15 had the lower amount of peptides with Gln4, but with a percentage very similar to DR1 and DR10.

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

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Figure legends

Figure 1. Two examples of the comparison of the DR1-, DR4-, DR10- and DR15-bound peptides bound by LC-MALDI-TOF/TOF MS. A) Fraction N. G16 was compared between the different allotypes. The peak with the mass 1721.9 corresponded to the peptide RVEYHFLSPYVSPK, as sequenced by MALDI-TOF/TOF. 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 closest masses, 1730.9 and 1726.9, are labeled in DR4 and DR15, respectively. **B) Fraction N. J16 was compared between the different allotypes.** The peak with the mass 1436.8 corresponded to the peptide QQYLPLPTPKVIG, as sequenced by MALDI-TOF/TOF. 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 closest masses, 1446.7 and 1391.8, are labeled in DR4 and DR15, respectively.

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

Table 1. Percentage of common molecular masses between the different haplotypes detected by LC-MALDI-TOF.

	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

Table 2. Common peptides associated to 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	Ragulator complex protein LAMTOR1	X	X		
APSTY AHLSPAKTPPP	Lipolysis-stimulated lipoprotein receptor	X	X		
APSTY AHLSPAKTPPPP	Lipolysis-stimulated lipoprotein receptor	X	X		
APSTY AHLSPAKTPPPPA	Lipolysis-stimulated lipoprotein receptor	X	X		
ASAF AFLAPARAIL	Solute carrier family 23 member 2	X	X		
DARSYTA AVANRAKGGG	Myotubularin-related protein 4	X	X		
DFYKILGVPRSASIK	DnaJ homolog subfamily B member 11	X	X		

IEKFEKEAAEMGKG	Elongation factor 1-alpha 1	X	X		
ISQELEELRAEQQL	Low affinity immunoglobulin epsilon Fc receptor	X	X		
KGRLDYLSSLKVKGLV	4F2 cell-surface antigen heavy chain	X	X		
LPTSFGSLAPAPLVFPGP	E3 ubiquitin-protein ligase RNF167	X	X		
LPVILDIKGECSRPG	Proactivator polypeptide	X	X		
NPKTFFILHDINSDG	Nucleobindin-1	X	X		
QQYLPLPTPKVIG	Heat shock 70 kDa protein 13	X	X		
QQYLPLPTPKVIGID	Heat shock 70 kDa protein 13	X	X		
QYLPLPTPKVIGID	Heat shock 70 kDa 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		

VDKVIAQQTAFSANPA	Syntenin-1	X	X		
DDTQFVRFDSDAASPR	HLA class I histocompatibility antigen, B-14 alpha chain		X	X	
VDDTQFVRFDSDAASPR	HLA class I histocompatibility antigen, B-14 alpha 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	Desmoglein-2	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	Dipeptidyl peptidase 1			X	X

Table 3. Theoretical cores of the common peptides associated to HLA-DR molecules and P4 residues used by them.

PEPTIDE BINDING CORE*	HOM-2 (DR1)	BEN (DR10)	WT51 (DR4)	MGAR (DR15)	P 4
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 ¹	X	X			L or I
YHF L SSPKE	X	X			L
FSQ L SSEHG	X	X			L

IQA Q TAFSA	X	X			Q
VRFD S DAAS		X	X		D
YRI E SVLSS		X	X		E
FYML L KGKLQ	X			X	L
VIAL L AGNKA or IVIAL A LAGNK ²	X			X	L or A
FVK A INAIQ or VKAI I NAIQK ³			X	X	A or I

*Cores were selected using the NetMHCIIpan 3.1 Server.

¹ FLAILGGAK to DRB1*10:01.

² IVIALAGNK to DRB5*01:01.

³ VKAINAIQK to DRB5*01:01.

Table 4. Experimental binding affinity of shared peptides associated to 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 IV TERSVSSRQA	8.26	0.33	3.36	0.55	>50
DR1-DR10	APST Y AHLSPAKTPPP	0.15	>50	0.09	>50	18.15
DR1-DR15	SPS I V I ALAGNKADL*	0.19	>50	>50	>50	<0.05
DR4-DR15	DHN F VKAINAIQKS	>50	0.04	1.75	<0.05	0.07
-	CONTROLS	0.07	0.16	0.93	0.05	0.1

The theoretical binding core is highlited in bold.

*The theoretical core for DRB5*01:01 is IVIALAGNK as defined by NetMHCII-pan3.1.

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