JLB

Article

Analysis of the HLA-DR peptidome from human dendritic cells reveals high affinity repertoires and nonconventional pathways of peptide generation

M. Teresa Ciudad, Nicoletta Sorvillo,[†,‡] Floris P. van Alphen,[†] Diego Catalán,[§] Alexander B. Meijer,[†,¶] Jan Voorberg,[†] and Dolores Jaraquemada*,[1]*

*Department of Cell Biology, Physiology and Immunology, Laboratori d'Immunologia Cellular, Institut de Biotecnologia i Biomedicina, Universitat Autònoma de Barcelona, Barcelona, Spain; [†]Department of Plasma Proteins, Sanquin-AMC Landsteiner Laboratory, Amsterdam, The Netherlands; [‡]Department of Pediatrics, Harvard Medical School, Boston, Massachusetts, USA; [§]Programa Disciplinario de Inmunología, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Santiago, Chile; and [¶]The Netherlands and Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, The Netherlands

RECEIVED FEBRUARY 5, 2016; REVISED MAY 24, 2016; ACCEPTED JUNE 12, 2016. DOI: 10.1189/jlb.6HI0216-069R

ABSTRACT

Dendritic cells (DCs) are the major professional APCs of the immune system; however, their MHC-II–associated peptide repertoires have been hard to analyze, mostly because of their scarce presence in blood and tissues. In vitro matured human monocyte-derived DCs (MoDCs) are widely used as professional APCs in experimental systems. In this work, we have applied mass spectrometry to identify the HLA-DR–associated self-peptide repertoires from small numbers of mature MoDCs (~5 × 10^6 cells), derived from 7 different donors. Repertoires of 9 different HLA-DR alleles were defined from analysis of 1319 peptides, showing the expected characteristics of MHC-II–associated peptides. Most peptides identified were predicted high binders for their respective allele, formed nested sets, and belonged to endo-lysosomal pathway-degraded proteins. Approximately 20% of the peptides were derived from cytosolic and nuclear proteins, a recurrent finding in HLA-DR peptide repertoires. Of interest, most of these peptides corresponded to single sequences, did not form nested sets, and were located at the C terminus of the parental protein, which suggested alternative processing. Analysis of cleavage patterns for terminal peptides predominantly showed aspartic acid before the cleavage site of both C- and N-terminal peptides and proline immediately after the cleavage site in C-terminal peptides. Proline was also frequent next to the cut sites of internal peptides. These data provide new insights into the Ag processing capabilities of DCs. The relevance of these processing pathways and their contribution to response to infection, tolerance induction, or autoimmunity deserve further analysis. *J. Leukoc. Biol.* 101: 15–27; 2017.

Introduction

Immature DCs internalize exogenous material via nonspecific processes (macropinocytosis), receptor-mediated endocytosis (FcγRs and lectin receptors), or phagocytosis of pathogens or apoptotic cells for Ag presentation by MHC-II molecules. They can also present endogenous material after its degradation by autophagy or by other unknown pathways. After activation, mature DCs mobilize peptide–MHC-II complexes to the surface and increase their stability to improve recognition by $CD4^+$ T cells and induce adaptive immune responses. The mechanisms that underlie the role of DCs in Ag presentation by MHC-II have been recently reviewed [1].

MHC-II αβ-dimers are synthetized into the ER where they associate to Ii trimers to prevent the binding of peptides inside the ER, forming nonameric $(\alpha\beta\text{-Ii})_3$ complexes. Once these complexes reach the endocytic pathway–associated Ag processing compartments, directly or after expression at the cellular membrane, Ii is sequentially degraded until only a small fragment (CLIP) remains bound to the MHC-II cleft. With the help of the chaperone HLA-DM, CLIP is released to allow the binding of antigenic peptides [2]. A low pH and reducing conditions that favor both Ii processing and Ag degradation characterize the proteolytic environment of the Ag processing compartments. Some of the most relevant proteolytic enzymes in these compartments are cysteine proteases (cathepsin B, F, H, L, S, Z, and the asparagine-specific endoprotease), serine

Abbreviations: DC = dendritic cell, ER = endoplasmic reticulum, HB = high binder, IB = intermediate binder, Ii = invariant chain, LB = low binder, MHC-I = MHC class I, MHC-II = MHC class II, MoDC = monocyte-derived dendritic cell, MS = mass spectrometry, PFR = peptide flanking residues

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

1. Correspondence: Institute of Biotechnology and Biomedicine. Universidad Autónoma de Barcelona, 08193, Barcelona, Spain. E-mail: dolores.jaraquemada@uab.cat

(cathepsin A and G), and aspartate proteases (cathepsin D and E). In addition, the IFN-γ–inducible lysosomal thiol reductase has been reported to reduce protein disulfide bonds, which generates partially denatured proteins and, thus, favors Ag degradation [3].

For the last 25 y, study of MHC-II peptide repertoires has been used to describe the allele-specific peptide binding motifs of human (HLA-DR, HLA-DQ, and HLA-DP) and mouse (IA and IE) MHC-II molecules and to analyze the general and the specific mechanisms of Ag processing and presentation. From the beginning, tumor or EBV-transformed B lymphoblastoid cell lines were used for mouse and human studies. In early reports, the relative inefficiency of sequencing methods was compensated by the use of very high numbers, usually between 10^8–10^{10} cells, to isolate the peptide-MHC complexes [4–9]. Other approaches, such as cells transfected with MHC-II molecules, have been used to study the importance of accessory molecules in the generation of peptide repertoires [10, 11]. An interesting comparison of MHC-II repertoires from human T cell clones and B lymphoblastoid cell lines that were derived from peripheral blood of the same donors showed that only ~10% of peptides belonged to cell type–specific proteins, whereas the remaining peptides were common to both cell types [12].

In addition, MHC-II peptide repertoires have been analyzed from several lymphoid tissues and primary cell cultures. MHC-II peptidomes from thymus and spleen have been described for both human [13] and mouse [14]. From our work on the thymus HLA-DR peptidome, we were first to report peptides derived from tissue-restricted Ags related to autoimmune diseases [15]. Mouse MHC-II peptide repertoires have also been studied from spleen B cells and activated macrophages [16, 17]. For DC studies, the high numbers of cells needed and the moderate yield of peptides obtained have been a difficult barrier to overcome. Previous data are available on MHC-II peptide repertoires from DCs in mouse, sheep, and human. In vivo–enriched splenic mouse DCs (2–5 × 10^8 cells) were used to study their IA/IE-associated peptidome [16] as well as the HLA-DR1 peptidome from transgenic mice [18]. In our study [19], 2–3 × 10^8 sheep DCs migrating from skin to draining lymph nodes were collected via cannulation of the pseudo-afferent lymph duct, which yielded a small number of sheep peptides. In human, a total of 115 nonredundant HLA-DR–associated peptides were obtained from thymus-resident DCs, isolated from 4 human thymus samples that yielded 30–66 × 10^6 DCs each [20]. Recently, by using 1.2 × 10^9 cells of a human monocytic cell line differentiated to DCs, a comprehensive HLA-DR (HLA-DR10/HLA-DR11) peptide repertoire was described [21].

Human in vitro MoDCs have been widely used as the best cellular model for professional APCs; therefore, the peptide repertoires presented by their MHC-II molecules are expected to be the gold standard for epitope identification. To date, small numbers of MoDCs have been used to identify HLA-DR peptides for different purposes. To analyze the influence of CLIP in the HLA-DR repertoire of immature and mature MoDCs, >200 HLA-DR4–associated peptides were isolated from 5 × 10^6 cells [22]. Later works used a similar approach to study the presentation of Ags involved in hemophilia A and thrombotic thrombocytopenic purpura [23–26]. In this work, we have applied the same methodology to MoDCs to refine the characterization of peptidomes presented by different HLA-DR alleles.

MATERIALS AND METHODS

mAbs

mAb anti–HLA-DR was purified from L243 hybridoma (American Type Culture Collection, Wesel, Germany) supernatant via protein A-sepharose and coupled at a final concentration of 5 mg/ml to CNBr Sepharose 4B (GE Healthcare, Buckinghamshire, United Kingdom). Abs anti–CD83-allophycocyanin, anti–CD86-allophycocyanin, anti–CD206-allophycocyanin (mannose receptor 6; BD Biosciences, San Jose, CA, USA), anti–CD209-phycoerythrin (DCSIGN; AbD Serotec, Düsseldorf, Germany), and anti–CD14-phycoerythrin (Sanquin Reagents, Amsterdam, The Netherlands) and their corresponding isotype controls were used for phenotype studies.

Blood samples and generation of mature MoDCs

Blood was drawn from HLA-typed healthy volunteers in accordance with Dutch regulations and after approval from the Sanquin Ethical Advisory Board in accordance with the Declaration of Helsinki. PBMCs were isolated from freshly drawn, EDTA anticoagulated blood by separation over a Ficoll-Paque PLUS gradient (GE Healthcare). Monocytes were purified by magnetic separation with anti-$CD14^+$ magnetic beads (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) and differentiated into immature DCs by culturing them in the presence of 1000 U/ml IL-4 and 800 U/ml GM-CSF (CellGenix, Freiburg, Germany). After 5 d, immature MoDCs were maturated by using 1 μg/ml LPS (Sigma-Aldrich, St. Louis, MO, USA) for 24 h in the presence of 1% human serum. Adherent maturated MoDCs were detached with PBS and washed before analysis.

Analysis of the cell surface phenotype by flow cytometry

Monocyte, immature MoDCs or mature MoDCs were washed with TBS that contained 0.5% HSA (Sanquin Reagents). Cells were incubated with 50 μl of 1 μg/ml mAb or appropriate isotype controls diluted in TBS/0.5% HSA for 30 min at 4°C. Cells were washed twice and resuspended in TBS/0.5% HSA. Cells were analyzed on a Fortesa flow cytometer (BD Biosciences) and with FlowJo (version 8.6; Tree Star, Ashland, OR, USA).

Confocal microscopy

Cells were washed with TBS/0.5% HSA and fixed in 4% paraformaldehyde in TBS for 15 min. Then, 50 mM NH_4Cl in TBS/0.2% saponin was added for 15 min to quench unspecific fluorescence. Fc receptors were blocked (human FcR blocking reagent; MACS; Miltenyi Biotec) at 4°C overnight. Cells were incubated with 1 μg/ml L243 Ab for 45 min at 4°C and the anti-mouse IgG2a Alexa Fluor 568–conjugated secondary Ab for 30 min at 4°C. Cells were mounted with Mowiol-Hoechst (Polysciences, Warrington, PA, USA). Preparations were visualized in a Leica TCS SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany)

Purification of peptide–HLA-DR complexes

Peptide–HLA-DR complexes were purified as described [23–26]. Cell pellets of 5 × 10^6 mature MoDCs were resuspended in 50 mM Tris-HCl (pH 7.0) that contained 4% MS-grade NP-40 (Thermo Fisher Scientific, Waltham, MA, USA) and protease inhibitor cocktail (Halt Protease and Phosphatase Inhibitor cocktail, EDTA free; Thermo Fisher Scientific) by end-over-end incubation at 4°C for 1 h. Cell lysates were cleared by centrifugation for 15 min at 4°C at 14,000 rpm. Peptide–HLA-DR complexes were purified from the soluble fraction by immunoaffinity chromatography using L243-coupled CNBr Sepharose 4B in overnight incubation at 4°C. Subsequently, L243 Sepharose was washed 3 times with 10 mM Tris-HCl, pH 7.0, that was supplemented with protease inhibitor cocktail and 5 times with 10 mM

Tris-HCl, pH 7.0, without protease inhibitor cocktail. Peptides were eluted from HLA-DR by adding 10% acetic acid for 15 min at 70°C. In parallel experiments, cell lysates were incubated with noncoupled CNBr Sepharose 4B to identify nonspecific-bound peptides.

MS analysis

Eluted peptides were purified from the acetic acid eluate and desalted by using C18 stage-tips prepared in-house (3M, Neuss, Germany) and then separated by using a reverse-phase C18 column made in-house from a silica tip emitter (New Objective, Woburn, MA, USA) that was filled with 1.9 μm C18 particles (Dr. Maisch, Ammerbuch-Entringen, Germany) at a flow rate of 300 nl/min, with a gradient from 0 to 80% (vol/vol) acetonitrile with 1% HAc. Separated peptides were sprayed directly into the LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) by using a nanoelectrospray source with a spray voltage of 2.1 kV. A collision-induced dissociation was performed for the 5 most intense precursor ions selected from each full scan in the Orbitrap (350–2000 m/z, resolving power 60,000). An isolation width of 2 Da was used for the selected ions (charge ≥ 2) with an activation time of 30 ms. Dynamic exclusion was activated for the MS/MS scan with a repeat count of 1 and exclusion duration of 60 s.

Database search and peptide identification

Peptides were identified on the basis of the MS/MS fragmentation spectra in a Sequest search algorithm against the UniprotKB human nonredundant protein database 25.H_sapiens.fasta by using Proteome Discoverer (version 1.4; Thermo Fisher Scientific). Search parameters allowed a peptide mass tolerance of 10 ppm, a fragment tolerance of 0.6 Da, no enzyme restriction, and variable modifications for oxidized methionine (+16 Da). A false discovery rate of 1% was used as filter. Peptides that did not comply with these criteria were excluded.

Theoretical binding assignment

Three evaluation methods were used to assign each peptide to the corresponding allele: ProPred [27], NetMHCIIpan [28], and an in-house software, Laia Motifs [29], on the basis of the binding motifs published in SYFPEITHI database (www.syfpeithi.de), except for HLA-DR10 (DRB1*1001) [30], HLA-DR4 (DRB1*0401) [10], and HLA-DR3 (DRB1*0301) (unpublished results), for which the binding motif was revised in our laboratory. This methodology has been previously tested experimentally [13]. NetMHCIIpan tool uses an artificial neural network that takes into account the residues in the anchor pockets of MHC-II molecule as well as the peptide core and flanking residues. With this system, HB peptides (corresponding to $IC_{50} < 50$) and IBs ($50 < IC_{50} < 500$) were assigned to 1 of the 2 DRB1-expressed alleles of each sample. The ProPred tool contains a database of binding matrices for 51 HLA-DR alleles and assigns a core sequence on the basis of the presence of at least a correct P1 residue according to the allele's binding motif and a low or high threshold depending on the other anchor residues (generally P4, P6, P9). The threshold is defined as the percentage of best scoring natural peptides. We used the following criteria: a peptide was considered HB if a core was assigned to the alleles at threshold ≤ 3 and IB if a core was assigned at a threshold between 9 and 3. For validation, we performed a manual analysis on the basis of the described allele-binding motifs. All possible 9-residue cores were identified from each sequence by fixing the P1 residue for each allele's motif. From the resultant cores, we chose the one that best complied with an allele-binding motif on the basis of the rest of positions. A core with ≥3 coincidences with the motif was considered HB, 2 coincidences were IB, and the remaining sequences, LBs. Finally, to assign a peptide to a given allele, at least 2 of the 3 methods must define the same binding core for each peptide and the same degree of affinity for the allele. If >1 core was acceptable, the one with higher affinity was considered. If a peptide could be associated to 2 alleles with the same affinity, it was noted as double-binder. Approximately 5% of peptides were not assigned as a result of discrepancy between the 3 methods.

Statistical analysis

Statistical analysis was performed by using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA). Variance was calculated with the 2-way ANOVA method followed by Bonferroni correction. A P value <0.05 was considered significant.

RESULTS

Characteristics of HLA-DR–associated peptide repertoires in mature MoDCs

Mature MoDCs from HLA-typed healthy donors were used to analyze their endogenous HLA-DR–associated peptidome from both exposed and internal MHC-II molecules. Phenotype of immature and mature MoDCs was analyzed by flow cytometry and confocal microcopy (**Fig. 1**). Only bona fide identifiable

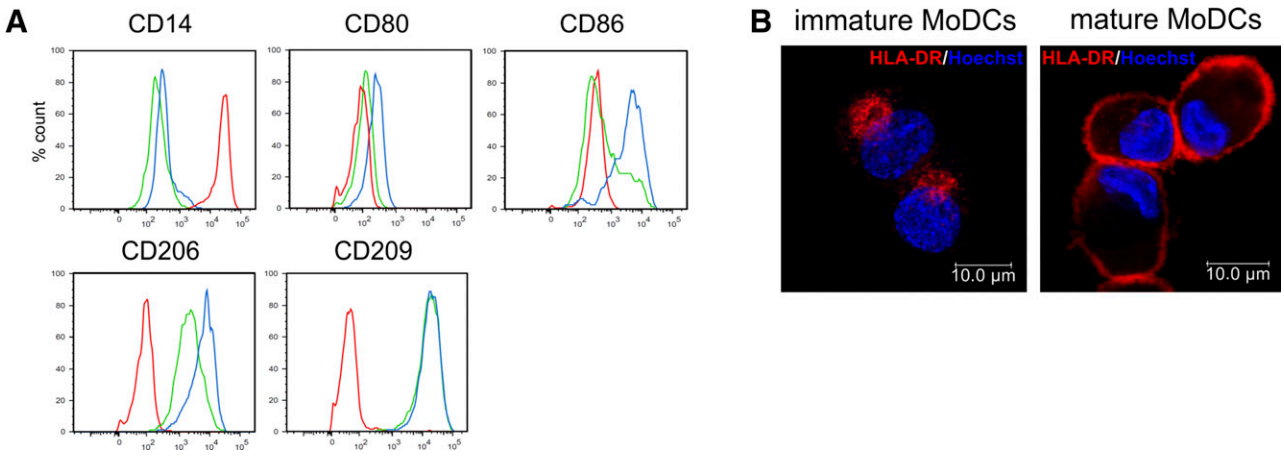

Figure 1. Phenotype analysis of human monocytes and immature and mature MoDCs. (A) FACS histograms represent the surface expression of CD14, CD80, CD86, CD206 (mannose receptor 6), and CD209 (DC-SIGN) by monocytes (red line), immature MoDCs (green line), and mature MoDCs (blue line). (B) Immunofluorescence detection of HLA-DR molecules (L243 Ab) and nucleus (Hoechst) in immature and mature MoDCs. HLA-DR molecules are retained in intracellular compartments and delivered to the cell surface after the maturation stimulus.

sequences and nonredundant peptides were included in the analysis. Thus, peptides derived from skin proteins (e.g., keratin, melanoregulin, and filaggrin) as possible handling contaminations, peptides from proteins nonspecifically bound to sepharose controls (including all peptides with the same binding core), sequences unidentifiable in databases, and redundant peptides were discarded (Supplemental Table 1). The complete list of accepted sequences, their source protein, and its most likely cellular localization are shown in Supplemental Table 2.

Analysis of HLA-DR–associated peptides from mature MoDCs that were derived from 7 HLA-DR–typed donors yielded 1319 peptides. Donor G typed DRB1*0701 and DRB1*1501. In the DRB1*1501 haplotype the HLA-DRB5*0101 gene is expressed, which generates a second HLA-DR molecule (HLA-DR51) that is as capable to present peptides with a well-defined motif as those encoded by DRB1 [31]; therefore, HLA-DR51 was also included within the analyzed molecules. To increase the number of peptides associated to each allele, donor G samples were prepared and analyzed by MS in 2 parallel experiments and the resulting peptides were then pooled.

Peptide analysis data are summarized in **Table 1**. Peptide size followed a normal distribution (**Fig. 2A**), with an average length of 16 residues as described for peptides associated to human MHC-II alleles [32]. Another classic feature of MHC-II–associated peptides is that they are often clustered in nested sets, that is, peptide families with a common binding core sequence but different length along C and N termini, which allows long peptides to bind MHC-II molecules [32]. In our analysis, between 44 and 73.5% of peptides were grouped in nested sets comprised of 2–20 peptides, whereas only a single variant was found for an average 42% peptides.

Between 85 and 362 peptides were analyzed per sample. They all derived from a total of 353 proteins, from which many were shared by ≥2 samples. Only 5 proteins were common to all samples: HSA, present in human serum used for cell culture medium supplementation, serine-tRNA ligase, 60S ribosomal protein L22, pro-LDL receptor-related 1, and low-affinity IgFcε receptor. Proteins related to Ag processing and presentation, such as HLA-DRα chain, HLA-Bα chain, and cathepsin B were found in >5 samples. Peptides from DCs and other myeloid cell–specific proteins, such as myeloperoxidase and the macrophage mannose receptor, were shared by 6 of 7 samples. CLIP peptide was found in samples from donors A, F, and G. Other Ii-derived peptides were identified from the same donors and donor D. Peptides from common proteins were abundant; for instance, 31, 20, 15, and 11 peptides from serum albumin were identified from donors E, D, G, and A samples, respectively. Annotated spectra of 11 representative peptides from these proteins are shown in Supplemental Fig. 1.

Location and function of the 353 parental proteins were determined on the basis of their annotation in the Gene Ontology database [33]. Source proteins represented a variety of cellular processes (Fig. 2B). As expected, ubiquitously expressed proteins that belonged to processes, such as basic metabolism, cell proliferation, or gene expression, were abundant. A wide range of proteins related to immune response (22%) were also identified, mostly MHC-I and -II Ag presentation-related proteins and molecules from the immune system signaling pathways.

Mature MoDCs mostly present predicted high-affinity peptides from the endo-lysosomal pathway

A methodology that combines 2 well-defined online prediction tools (ProPred and NetMHCIIpan) and an in-house software (Laia Motifs) was used to assign each peptide to the corresponding allele (see Materials and Methods). Our 3 method–based prediction was previously tested experimentally. Results of this conservative prediction approach were all coincident when experimental high-affinity peptides were compared, whereas predicted LBs and IBs did not always coincide with experimental data [13].

Because of the large size of MHC-II peptides, ≤46% were assigned to >1 allele in some of the samples and most of them were HBs for both alleles. For HLA-DR1– or HLA-DR4–positive donors C, D, and E, there was a clear prevalence of peptides assigned to these alleles (47.2, 47.5, and 68.3%, respectively) *vs.* partner alleles (17.9, 5, and 5.7%, respectively) when discarding double-binder peptides. Looking to peptides exclusively associated to 1 allele from HLA-DR15–positive donor G, some peptides were assigned to HLA-DR51 (DRA1*0101/DRB5*0101), which confirmed the contribution of DRB5 alleles to the HLA-DR peptide repertoires.

To study the predicted affinity of analyzed peptides for each HLA-DR allele in the context of professional APCs, we pooled all peptides from different samples assigned to the same allele. Double-binder peptides were included for the analysis of both alleles. An average of 76% peptides were predicted HBs for their

TABLE 1. Characteristics of MoDCs samples and description of peptides and source proteins

Characteristic	Donor A	Donor B	Donor C	Donor D	Donor E	Donor F	Donor G
HLA-DR type	DRB1*0301 DRB1*1101	DRB1*0301 DRB1*1301	DRB1*0401 DRB1*1301	DRB1*0101 DRB1*0701	DRB1*0101 DRB1*1101	DRB1*0901 DRB1*1001	DRB1*0701 DRB1*1501 DRB5*0101
Nonredundant peptides (*n*)	140	85	106	219	194	213	362
Proteins (*n*)	79	57	70	98	89	85	138
Unique peptides, *n* (%)	66 (47.1)	47 (55.3)	70 (66)	69 (31.5)	74 (38.1)	65 (30.5)	96 (26.5)
Peptides in nested sets, *n* (%)	74 (52.5)	38 (44.7)	36 (44)	150 (68.5)	120 (61.9)	148 (69.5)	266 (73.5)
Nested sets	25	16	12	39	35	44	80
Peptides per protein (range)	1–11	1–6	1–5	1–20	1–31	1–14	1–21
Peptides per nested set (range)	2–7	2–6	2–5	2–20	2–20	2–14	2–15

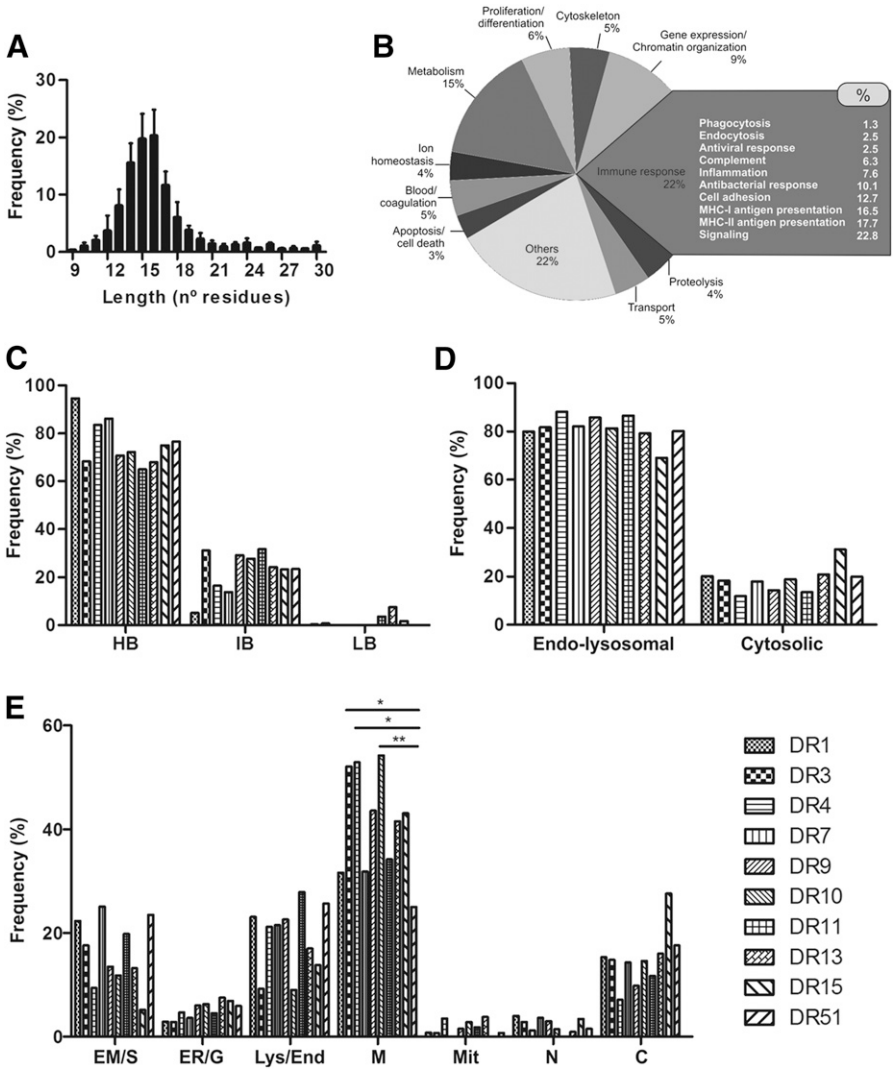

Figure 2. Size, functional distribution, and degradative pathway and location of HLA-DR–associated peptides (n = 1319) in mature MoDCs. (A) Size of MHC-II peptides followed a normal distribution, with an average size of 16 residues. Bars represent average peptide frequency (%) ± SD from each donor. (B) Functional clustering of the parental proteins (n = 353) on the basis of the annotation in Gene Ontology Database. (C) Theoretical affinity assignment. Peptides assigned to the same allele were pooled together, independent of the donor. Double-binder peptides were included in the analysis of both alleles. Approximately 5% of all peptides were not assigned to any allele as a result of a discrepancy in the analysis. For the rest, an average of 76% were theoretically assigned as HBs, 22.6% as IBs, and 1.4% as LBs. Bar graph represents assigned peptide frequency for each allele. (D) Degradative pathway of the HLA-DR–associated peptide parental proteins per allele. Endo-lysosomal degradation pathway included proteins from cell membrane (M), extracellular matrix or secreted proteins (EM/S), endoplasmic reticulum/Golgi (ER/G), and lysosome/endosome (Lys/End). Mitochondrial (Mit), cytosolic (C), and nuclear (N) proteins were included as the cytosolic pathway of degradation. (E) Subcellular location of the parental proteins. The contribution of membrane components to the HLA-DR peptidome is significantly lower in HLA-DR51 (HLA-DRB5*0101) peptides compared with HLA-DR4, HLA-DR10, and HLA-DR3. Bars represent peptide frequency for each allele. Two-way ANOVA; Bonferroni post-test $^*P < 0.05$; $^{**}P < 0.01$.

respective allele (Fig. 2C), whereas >22.6% were assigned as IB, and 1.4% were LBs. We did not find significant differences in predicted affinity between alleles.

In a standard location definition, secreted and extracellular matrix proteins, ER and Golgi apparatus, lysosomes/endosomes and cellular membrane component proteins are expected to be degraded in the endo-lysosomal pathway for Ag processing and presentation by MHC-II molecules. Instead, mitochondrial, cytosolic, and nuclear proteins are associated to the cytosolic pathway. As expected for MHC-II peptides [8, 34], the endo-lysosomal pathway was predominant (~80% of peptides) over the cytosolic pathway (~20%; Fig. 2D). When focused on the specific compartments, the cellular membrane was shown to be the main source of proteins, followed by the extracellular or secreted proteins and lysosomal/endosomal components (Fig. 2E). This was expected in cells that were derived from cell culture conditions, where the extracellular milieu only contains serum and some secreted proteins. Of interest, even in these culture conditions, significant differences were observed for HLA-DR51 compared with other HLA-DR molecules. Only 25% of peptides were derived from the cellular membrane, which was as many as from the extracellular milieu and the lysosome/endosome proteins.

PFRs are restricted in some HLA-DR alleles

PFRs of the MHC-II peptides are defined as the residues adjacent to the binding core in their N and C terminus. PFRs may influence peptide binding to MHC and recognition by TCR [35, 36]. Peptide binding is mediated by hydrogen bonds between the side chain of these residues and residues located at the MHC-II α and β chains. We analyzed PFR length for each HLA-DR allele repertoire. Because the length of the PFR is dependent on the assigned binding core, doubled-binder peptides were excluded from this particular study, but all members of each nested set were included. Analysis showed that the average length for N terminus PFRs was 4 residues, except for HLA-DR9 and HLA-DR51 peptides, where it was 5 and 3 residues, respectively. For C terminus PFRs, the average length was 3 residues, except for HLA-DR13–associated peptides, with an average of 4 residues, and HLA-DR9 and HLA-DR7 peptides, with 2 residues.

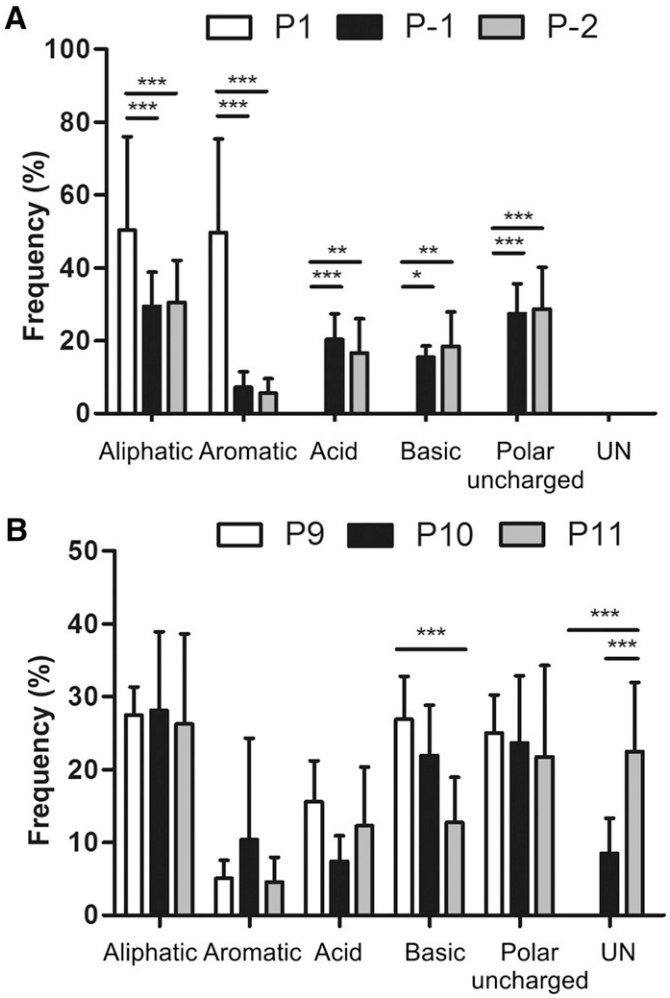

Figure 3. N and C terminus PFRs grouped by the physicochemical properties of their side group in aliphatic (G, A, V, L, M, I), aromatics (F, Y, W), polar-uncharged (S, T, C, P, N, Q), acid (D, E), or basic (H, K, R). (A) Anchor position P1 (white bars) were preferentially hydrophobic residues (aliphatic and aromatic), whereas in P-1 (black bars) and P-2 (gray bars), basic, acid, and uncharged polar residues were more abundant. (B) Similar preference for aliphatic, basic and uncharged polar amino acids was found in anchor position P9 and adjacent P10 and P11. UN indicates that no residue was found in that position. Bars represent average percent of each group ± SD. Two-way ANOVA; Bonferroni post-test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

To compare biochemical characteristics of peptide repertoires associated to each HLA-DR allele, amino acids were classified according to the physicochemical properties of their side chains into aliphatic (G, A, V, L, M, I), aromatic (F, Y, W), polar-uncharged (S, T, C, P, N, Q), acid (D, E), or basic (H, K, R). As expected, amino acids at the P1 anchor position were equally distributed into aliphatic and aromatic (**Fig. 3A**). Hydrophobicity is a shared feature for the residues that occupy the P1 anchor position of HLA-DR peptides [32]. For the N terminus PFR, a higher frequency of basic, acid, and polar amino acids in P-1 and P-2 compared with P1 was remarkable for most alleles. For example, 32% of HLA-DR15 peptides had Glu in P-1 and 33% had Lys in P-2. Other alleles, such as HLA-DR9 and HLA-DR51, also had preferred residues in both positions. For HLA-DR9, 29% of P-1 and 26% of P-2 residues were Val and Ser, respectively. In HLA-DR51, 27% Asn and 22% Val occupied P-1 and Gly constituted 27% of residues in P-2. These frequencies did not vary in relation to the predicted affinity of the peptides (data not shown).

For C terminus PFRs, anchor position P9 and the adjacent positions P10 and P11 were analyzed (Fig. 3B). As described above, C terminus PFRs were shorter than N terminus PFRs and ≤25% of the peptides were so short that there was no residue in position P11, especially for those assigned to HLA-DR9, HLA-DR3, HLA-DR13, and HLA-DR7. Residues that occupied all 3 positions were mostly aliphatic, basic, or polar-uncharged. HLA-DR9 seemed to be more restrictive at position P10 (28% Pro). HLA-DR15 also had a preference for Trp in P10 (25%) and Leu in P11 (25%). These data indicate that HLA-DR9 and HLA-DR15 are within the most restrictive alleles for both N and C terminus PFRs.

Peptides derived from the N- and C-terminal ends of the protein are preferentially generated from cytosolic and nuclear proteins

To determine the importance of the protein structure in peptide generation, we studied the location of the peptides along the parental protein sequence. Peptides located in the first 30 residues were considered N-terminal peptides and those located in the last 30 residues were considered C-terminal peptides. In mature MoDCs peptidomes, most peptides (82.4%) were located in the middle of the parental protein sequence (internal peptides; **Fig. 4A**), whereas N- and C-terminal peptides constituted an average of 5 and 12%, respectively. To note, all C-terminal peptides were located at the end of the protein, whereas only 25% of the N-terminal peptides began in the first or second residue of the protein. Most terminal peptides (62% N- and 76% C-terminal peptides) were unique sequences and corresponded to unique regions of the parental protein being presented, and most were HBs (Fig. 4B). Taking into account the Ag-processing route, defined by the subcellular origin of the parental proteins, the presentation of terminal peptides was favored for cytosol-degraded proteins (Fig. 4C).

A significantly reduced frequency of internal peptides processed by the cytosolic pathway was observed (Fig. 4D). Similar data were obtained when the HLA-DR peptide repertoire from thymus DCs [20] was reanalyzed. The published list of peptides was revised, redundant peptides from each sample were discarded, and, finally, 115 accepted peptides were subjected to the same analysis. Of interest, C-terminal peptides were again preferentially generated in the cytosolic degradation pathway (Fig. 4E).

Different cleavage motifs are found depending on peptide location in the protein

In contrast to MHC-I, the influence of individual proteases in the generation of MHC-II peptide repertoires has not been fully studied. To further understand the role of MHC-II pathway–associated proteases, we studied the amino acids in the first (N terminus) and last (C terminus) position of all nonredundant peptides after pooling all samples ($n = 1042$). Adjacent sequences

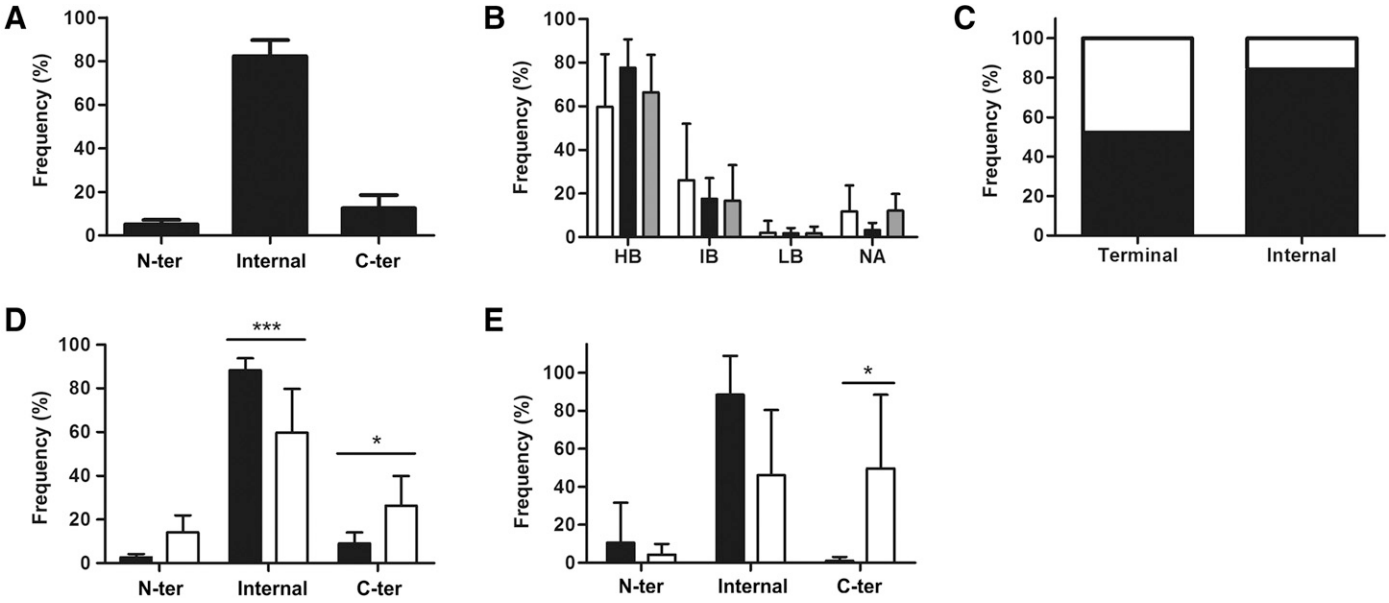

Figure 4. Peptide location in the parental protein sequence. The 30 first residues were considered the N-terminal (N-ter) end of the protein and the last 30 residues as the C-terminal (C-ter) end ($n = 1319$). (A) General overview showed that an average 82.4% of peptides were derived from the internal part of the proteins, whereas nearly 5% were N-ter peptides and 12% were C-ter peptides. (B) Theoretical affinity analysis of the N-ter peptides (white bars), internal peptides (black bars), and C-ter peptides (gray bars) showed no difference between terminal and internal peptides; the majority were predicted HB peptides independent of the location in the protein. (C) Contribution of the cytosolic pathway (white boxes) and the endo-lysosomal pathway (black boxes) to the generation of terminal or internal peptides. (D) Terminal peptides, especially C-ter peptides, were significantly favored in proteins degraded in the cytosolic pathway (white bars) compared with the endo-lysosomal pathway (black bars). (E) Reanalysis of the HLA-DR peptides from thymus DCs [20] according to the location of the peptide in the protein and the route of degradation ($n = 115$). As shown in MoDCs, C-ter peptides came preferentially from the cytosolic pathway (white bars) compared with the endo-lysosomal pathway (black bars). Bars represent average percent ± SD. Two-way ANOVA; Bonferroni post-test. $^*P < 0.05$; $^{***}P < 0.001$.

in their parental protein were also analyzed to try to identify ≥1 cleavage motifs. Following the literature for MHC-II pathway–related proteases [37], 4 residues were analyzed (R2, R1, R′1 and R′2) from each terminus and proteases were described to cut between R1 and R′1. Residues at the N terminus were referred to as NR, and CR for C-terminal residues. Amino acids were grouped as described above.

Different proteases should take part in Ag processing depending on the degradation route of the source protein: endo-lysosomal or cytosolic. Expected differences were observed in peptides that were derived from proteins presumably degraded by the endo-lysosomal pathway compared with cytosolic processing (**Fig. 5**, Supplemental Fig. 2A). For the generation of the N terminus side of the peptide (Fig. 5A), endo-lysosome–generated peptides significantly preferred Pro (17%) in position NR′2 and cytosolic peptides preferred Asp (20%) in the position immediately before the cut, NR1 (Fig. 5C). For C terminus generation, basic residues (22% Lys and 10.8% Arg) were preferred at CR1 in both pathways (Fig. 5B and D). Endo-lysosomal peptides frequently had Pro at CR2 (15.8%) (Fig. 5B).

Significant differences in cleavage motifs were also shown when the location of the peptide in the protein sequence was included in the analysis (**Fig. 6**, Supplemental Fig. 2B). Internal peptides showed a similar distribution of amino acids at their N- and C-terminal cleavage regions, where mostly aliphatic and uncharged polar amino acids were found (Fig. 6A and B). Data on terminal peptides showed some clear patterns. For the generation of N-terminal peptides, protease activity is focused on the C terminus of the peptide, the side from which the peptide would be released from the protein backbone. On one hand, in sequenced N-terminal peptides, positions CR2 and CR1 were markedly different from the rest, with an increment of Lys (22%) and Pro (20%) in CR2 and Asp (22%) in CR1 (Fig. 6C), with both residues located just before the cleavage site. On the other hand, for C-terminal peptides, protease activity is focused on the N terminus of the peptide. Sequenced C-terminal peptides showed a strong preference for Asp (48%) in the catalytic position NR1, whereas Pro was significantly high (30%) in the next residue, NR′1 (Fig. 6D). In addition, 20% of residues in NR′2 were Pro (Fig. 6D). Thus, these data indicate that Asp and Pro are relevant for cleavage site recognition by the proteases that generate terminal peptides. Although the double Asp-Pro motif was barely found, it must be pointed out that the acidic elution used in this method may have specifically disrupted this bond.

Databases show that HLA-DR peptides from cytosolic and nuclear proteins might be generated by both cytosolic and endo-lysosomal proteases

To further understand how peptides from cytosol and nuclear proteins end up being presented by HLA-DR molecules, we manually analyzed the generation of their specific cut sites by

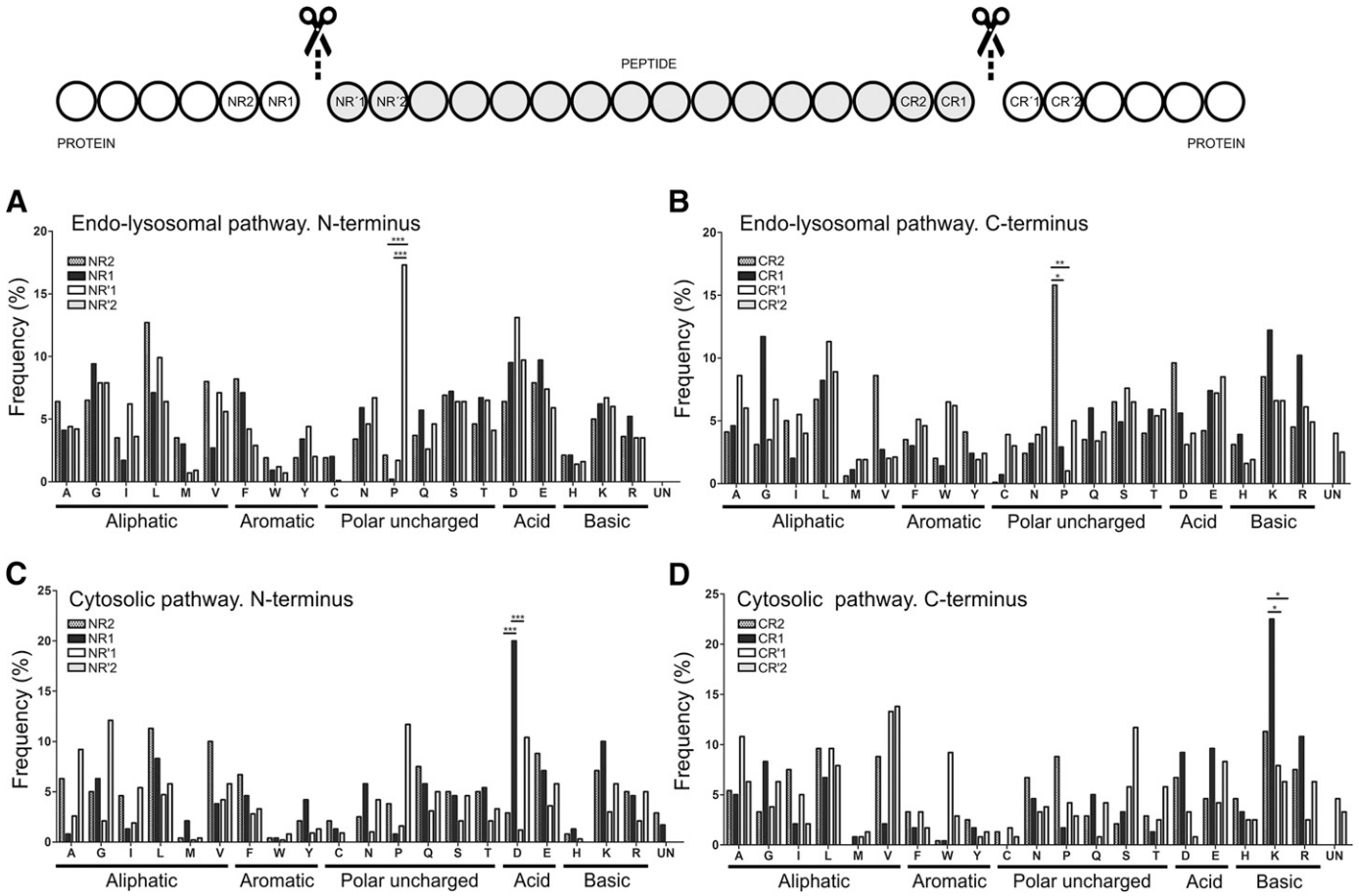

Figure 5. Amino acid frequency in the catalytic positions involved in N and C terminus generation of peptides degraded by the endo-lysosomal (n = 802; A and B) and cytosolic (n = 240; C and D) pathways of protein degradation. N terminus patterns were analyzed at positions NR2 (dotted bars), NR1 (black bars), NR′1 (white bars), and NR′2 (gray bars). C terminus patterns were analyzed at positions CR2 (dotted bars), CR1 (black bars), CR′1 (white bars), and CR′2 (gray bars). Bars represent the individual amino acid frequency (%). If no amino acid was found at a given position, it was counted as undefined (UN). Two-way ANOVA; Bonferroni post-test. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

using the databases MEROPS [38] and CutDB [39]. First, we looked at the information of protein cleavage for the parental proteins represented in our HLA-DR peptidome. MEROPS had data for 77.5% of the proteins compared with CutDB, which had records for only 26.2%. Proteases involved in the processing of these proteins were mainly caspases, calpains, cathepsins, granzymes, and matrix metallopeptidases. Most of these parental proteins have been reported to be degraded by both cytosol and endosome-related proteases.

In addition, we examined whether the location of the peptides in their parental protein (terminal or internal peptides) could be related to any of the degrading proteases. An average of 66% of parental proteins from each group were included in MEROPS database. There were no statistical differences in the distribution of proteases described to cleave these proteins (**Fig. 7A**). These data suggest that any of these of proteases could be involved in the generation of terminal peptides.

Only MEROPS contained enough information for the analysis of each peptide that was derived from cytosolic and nuclear proteins (n = 240). The specific bond cleavage of 5.8% (14 of 240) N-terminal peptides and of 0.83% (2 of 240) C-terminal peptides could be determined. Only proteases related to the endo-lysosomal degradation pathway (cathepsin D, cathepsin E, cathepsin L, cathepsin S, granzyme A, meprin α subunit, and matrix metallopeptidase-2) were described as putatively involved in the generation of these peptides (Fig. 7B).

DISCUSSION

In-depth analysis of human MHC-II peptide repertoires of human APCs has been an arduous task because an adequate number of cells were not easily obtainable. To date, the natural HLA-DR peptidome from human thymus DCs has been analyzed [20]. Thymus DCs isolated by their expression of CD11c were separated from other thymus cell subsets from 4 donors, and 115 nonredundant HLA-DR–associated peptides were identified. The starting material for the study was 30–66 $\times$ 10^6 DCs, yielding up to 48 peptides/sample, a big step in the identification of natural DC-presented peptides. Pending higher-efficiency methods to isolate DCs from thymus and other tissues, we and others have used different approaches to complete the characterization of Ag

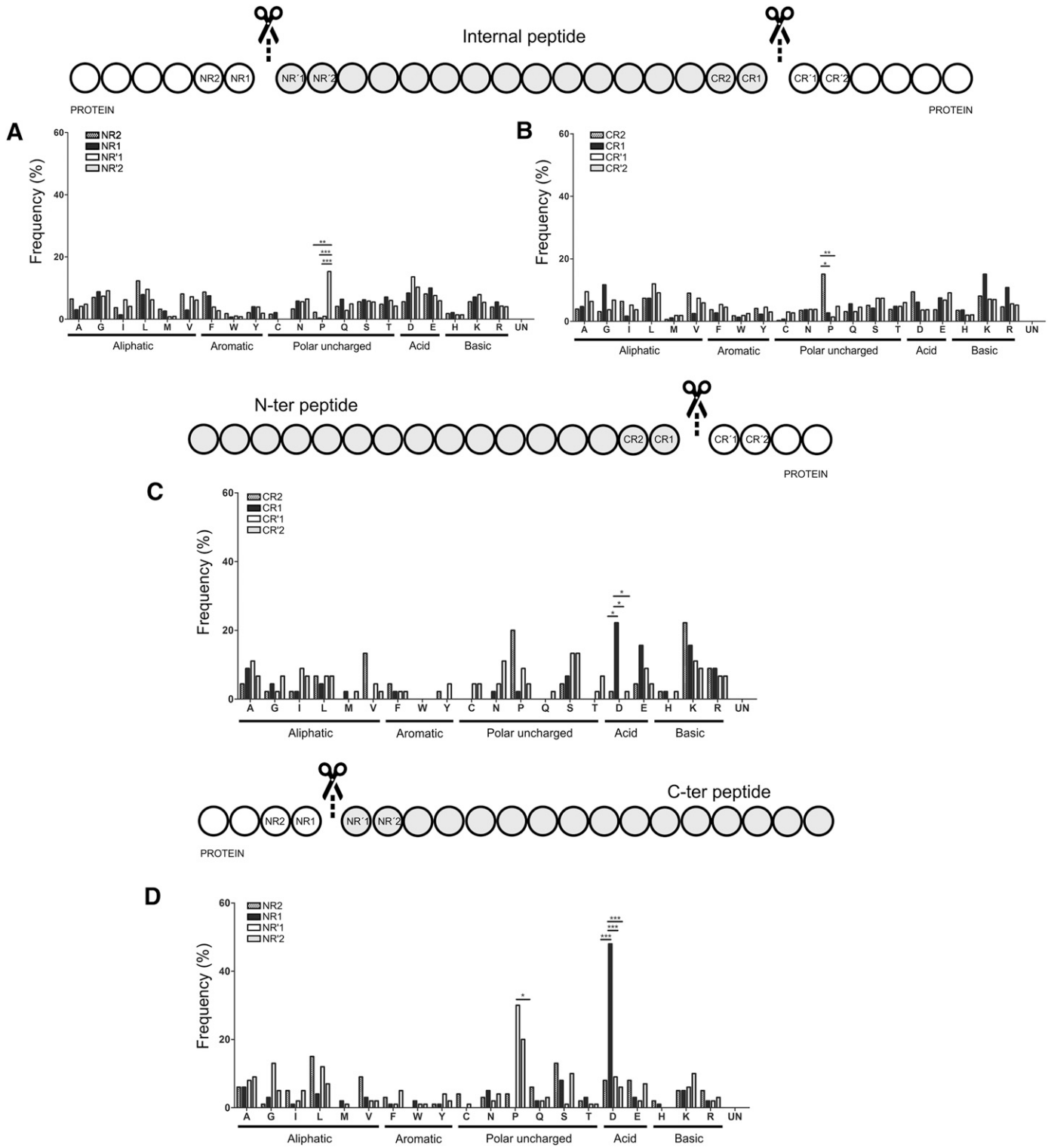

Figure 6. Amino acid frequency in the cleavage positions involved in N and C terminus generation of peptides according to their location in the parental protein. (A and B) Pattern of the internal peptides ($n = 897$) was studied for N terminus (A) and C terminus (B) generation. (C and D) N-terminal (N-ter) peptides ($n = 45$) were analyzed at their C-terminus ends (C) and C-terminal (C-ter) peptides ($n = 100$) were analyzed at their N-terminus (D). (A and D) N terminus pattern was analyzed at positions NR2 (dotted bars), NR1 (black bars), NR′1 (white bars), and NR′2 (gray bars). (B and C) C terminus pattern was analyzed in positions CR2 (dotted bars), CR1 (black bars), CR′1 (white bars), and CR′2 (gray bars). Data are represented as individual amino acid frequency (%). If no amino acid was found at a given position, it was counted as undefined (UN). Two-way ANOVA; Bonferroni post-test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

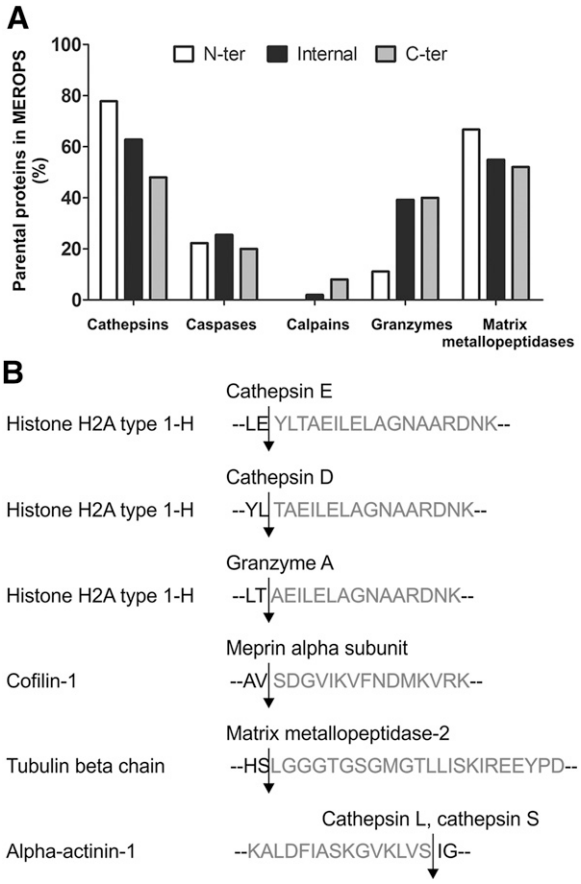

Figure 7. MEROPS analysis of the cleavage sites generation of the HLA-DR peptides derived from cytosolic and nuclear proteins. (A) Frequencies of the proteases described to cleave the parental proteins of the HLA-DR–associated peptides according to the location of the peptides in the protein sequence. Note: most parental proteins could be processed by multiple proteases. (B) Examples of the specific bond cleavage and the protease described to be involved in the generation of 5/14 N terminus ends and 1/2 C terminus ends of the isolated peptides (labeled in red). C-ter = C-terminal, N-ter = N-terminal.

processing and presentation by mature DCs. So far, the largest data set published has recently been obtained from the MUTZ-3 cell line after differentiation to DC in vitro, which yielded 14,000 peptides associated to 3 different HLA-DR molecules, HLA-DR10, HLA-DR11, and HLA-DR52 [21]. The work is very comprehensive, but the need for large numbers of cells was not overcome.

We instead used 5×10^6 mature MoDCs to identify HLA-DR–associated peptides. These cells are commonly used to analyze DC Ag processing but their natural peptidome was not fully defined. Here, we report the identification of 1319 peptides associated to 9 different HLA-DR alleles from mature MoDCs from 7 donors. Isolated peptides had standard size and were mostly grouped in nested sets (44–74%) as in thymus DCs (46–80%) [20] and in the MUTZ-3 peptidome (84%) [21]. The list of peptide source proteins was part of that identified by the proteomic profile of similarly matured MoDCs [40]. The later work by Mommen et al. [21] showed that peptides were only generated from 35% of the proteins that constituted the global MUTZ-3 proteome. In our hands, the number of proteins presented by most alleles was relatively low. Thus, the choice of source protein is limited by the presence of an adequate allele-binding motif in their sequence. An over-representation of proteins related to the immune system was found, mostly involved in MHC pathways and receptor signaling. A large proportion of source proteins that were identified in our study (40–55%) were also found in previous studies on DCs [20, 22]. Ii-derived CLIP peptides were found to be assigned to HLA-DR3, HLA-DR7, HLA-DR9, HLA-DR10, and HLA-DR51 molecules, but not to others, which confirmed allele-specific differences of HLA-DR–CLIP interaction. Expression of HLA-DR–CLIP complexes at the mature MoDC cell surface was reported to be increased compared with immature MoDCs [22].

MHC-II peptides are usually large and may bind >1 allele because their sequence may include several binding cores. Up to 46% peptides from our samples were assigned to both HLA-DR alleles expressed. A reanalysis of the published thymus DC peptidome data confirmed this and also that most self-peptides (~75%) were HBs for their corresponding alleles. This predominance of HBs was also reported from the MUTZ-3 peptidome [21]. As highly specialized APCs, DCs have an active machinery of Ag processing. When MoDCs were pulsed with Ag [23–26], most Ag-derived peptides were also HBs. A recent analysis of the HLA-DR1 peptidome of in vivo–enriched splenic DCs from transgenic mice also showed predicted high-affinity peptides to be the most abundant [18]; however, our prediction analysis of peptides from HLA-DR11, HLA-DR8, HLA-DR3, and HLA-DR4 lymphoblastoid cell lines [10–12, 41] and rat insulinoma cells transfected with HLA-DR4 [11] showed a different distribution, where the intermediate- and low-affinity peptides represented between 40 and 60% of the total repertoire. Thus, preference for high-affinity peptides may be cell type–dependent, which suggests that the intrinsic machinery of DCs may favor their generation and presentation, although a certain influence of the allele must be considered.

Peptide length and PFRs have been proposed to help in peptide stabilization and TCR interaction with the peptide–MHC-II complexes [35, 42, 43]. A published in silico analysis correlated peptide length with their experimental affinity by using data from 19 MHC-II allele ligands from the AntiJen database [44]. This analysis showed a point (~19 residues) beyond which higher peptide length did not result in higher affinity, although MHC-allele characteristics should also be taken into account. We looked at the number of residues that comprised the N and C terminus PFRs as determined by the position of the assigned allele-dependent binding core. Average size of the N-terminal PFR was 4 residues and 3 for C-terminal PFR, but small allele-dependent differences were observed. We also studied each amino acid frequency in PFRs, which were described as key positions for peptide stabilization [36, 45] but also for TCR recognition [35]. Previous studies that were centered on peptides from well-known Ags, such as HIV Gag (p24) or influenza HA, described the alterations in stability and T cell stimulation by peptide modifications affecting PFRs [46, 47]. Only 2 studies of HLA-DR repertoires focused on this particularity [48, 49]. The first study analyzed peptides that

belonged to the most abundant nested sets associated to HLA-DR4. The second work revealed an enrichment of acid residues and proline at the N terminus and the preference for basic residues at the C terminus of the flanking sequences. In contrast, our data showed that, in addition to a hydrophobic P1, there was a clear preference for reactive residues in the adjacent positions P-1 and P-2 that could interact with conserved HLA-DRα51 Phe, HLA-DRα53 Ser, and HLA-DRβ81 His residues via hydrogen bonds [45]. There also were differences in PFR amino acid frequencies when individual HLA alleles were compared, which showed that HLA-DR9 and HLA-DR15 were the most restrictive alleles at both sides (data not shown). These data contrast with those published by Godkin et al. [49], who reported a high homology between the PFRs of different alleles. The number of peptides analyzed may explain this difference.

As described, nearly 80% of HLA-DR–associated peptides were derived from the endo-lysosomal pathway of Ag processing and 20% from the cytosolic pathway. Autophagy and phagocytosis of material from dead or damaged cells is probably the most common provider of degraded cytosolic material to the endo-lysosomal pathway [34]. Databases MEROPS and CutDB only showed cleavage data for ≤5.8% of the peptides derived from cytosolic and nuclear proteins, mainly by endosome-related proteases. However, when taking into account the processing data of the entire parental protein, cytosol proteases, such as caspases and calpains, could also be involved. In our analysis of the cleavage pattern, peptides from cytosolic proteins showed high frequencies of Asp and Lys residues at N and C terminus, respectively, in contrast to peptides that were generated in the endo-lysosomal pathway, where Pro was increased. Of interest, cytosolic proteins are usually degraded by the immunoproteasome, which is known to cleave preferentially after hydrophobic and basic residues, such as Lys, but to a lesser extent after acidic residues [50]. As an advantage, terminal peptides can be generated by a single protease cleavage, which may explain their over-representation from cytosolic proteins. This suggests that these cytosolic parental proteins may have access to a non-conventional pathway of MHC-II Ag generation. To better understand the generation of these peptides, more experimental work must be done.

The vast majority of peptides were internal sequences of the parental proteins, which suggests that most peptides were generated in a late degrading milieu where partial degradation of the source proteins would already have happened. However, approximately 20% of peptides were directly derived from the N- and, mostly, C-terminal ends of the source protein. These terminal peptides were unique from their parental protein. Of interest, the proportion of terminal peptides from cytosol-degraded proteins was higher (40%) than that for endo-lysosome–degraded proteins (12%). In particular, the proportion of C-terminal peptides derived from cytosolic proteins was very large (62% of all terminal peptides). We had previously described a similar phenomenon in cells that expressed transfected HLA-DR in the absence of HLA-DM and Ii [11], where single peptides from the terminal regions of cytoplasmic proteins were eluted from HLA-DR4–transfected cells. In a recent work, while reviewing how immunodominant $CD4^+$ T cell epitopes were generated and selected [51], it was noted that several immunodominant peptides from Ags such as fibrinogen, MBP, GAD65, and cytochrome *c* were located at the N- or C-terminal ends of proteins.

C-terminal peptides also had a marked preference for Asp in the position before the cleavage site (R1), and most peptides suggested a possible cleavage pattern with Asp in position R1 and Pro in R′1. Asn and, to a lesser extent, Asp are allowed as precleavage residues for asparagine-specific endoprotease, although there are no data concerning the postcleavage residue [37, 52]. The contribution of the MHC-I Ag processing machinery may also be relevant in this context. Besides the eventual ability of immunoproteasome to cleave acidic residues [50], an MHC-I pathway–related protease, the signal peptide protease of the ER, also generates C-terminal peptides from transmembrane proteins [53]. Of interest, a bulk analysis of the peptidome from the MUTZ-3 cells also showed Asp in the adjacent positions of the cleavage side of peptides [21].

In addition, internal peptides showed some preferential cleavage residues, with Pro as a significantly predominant residue in both the N-terminal and C-terminal ends. The high frequency of Pro at the N-terminal position has been described in HLA-DR repertoires [21, 54]; however, terminal trimming must be considered and is consistent with the fact that internal peptides mostly belonged to nested sets [48, 55]. It is known that some aminopeptidases cannot cleave the Pro bonds and that Pro is an unfavorable cut residue for most MHC-II–related cathepsins [37]; therefore, the preferential presence of Pro at both ends of the internal peptides may be more related to its own capacity to stop cleavage by many proteases than to being the specific target of a single enzyme. This may not apply so much to C-terminal peptides as most did not form nested sets and were unique sequences.

Our current knowledge of the human MHC-II processing pathways for the efficient presentation of peptides is vast but still incomplete. The small numbers of cells used by our method may facilitate analyzing new processing pathways and monitoring different steps of human Ag presentation. Treatment of APCs with specific protease inhibitors, Ag stimuli, or stress inducers would help to elucidate new mechanisms involved in the generation of the MHC-II peptidome under physiologic and pathologic conditions, thereby providing insights into its role in tolerance induction, autoimmunity, and response to infection.

AUTHORSHIP

M.T.C. designed and performed experiments, analyzed data, and wrote the manuscript. N.S. designed and performed experiments. F.P.v.A. and A.B.M. conducted the proteomics analyses. D.C., J.V., and D.J. analyzed data and discussed the manuscript.

ACKNOWLEDGMENTS

This work was supported by a travel grant from the European Federation of Immunological Societies (to M.T.C.), by Grant SAF2012-35344 from the Spanish Ministry of Economy and

Competitiveness (to D.J.), and an Formación de Personal Investigador (FPI) fellowship to M.T.C. (BES-2010-030963). The authors thank Luis Ciudad for technical support in database analysis.

DISCLOSURES

The authors declare no conflicts of interest.

REFERENCES

1. Roche, P. A., Furuta, K. (2015) The ins and outs of MHC class II-mediated antigen processing and presentation. *Nat. Rev. Immunol.* **15,** 203–216.
2. Neefjes, J., Jongsma, M. L., Paul, P., Bakke, O. (2011) Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat. Rev. Immunol.* **11,** 823–836.
3. Van Kasteren, S. I., Overkleeft, H. S. (2014) Endo-lysosomal proteases in antigen presentation. *Curr. Opin. Chem. Biol.* **23,** 8–15.
4. Chicz, R. M., Urban, R. G., Gorga, J. C., Vignali, D. A., Lane, W. S., Strominger, J. L. (1993) Specificity and promiscuity among naturally processed peptides bound to HLA-DR alleles. *J. Exp. Med.* **178,** 27–47.
5. Falk, K., Rötzschke, O., Stevanović, S., Jung, G., Rammensee, H. G. (1994) Pool sequencing of natural HLA-DR, DQ, and DP ligands reveals detailed peptide motifs, constraints of processing, and general rules. *Immunogenetics* **39,** 230–242.
6. Rudensky, A. Y., Preston-Hurlburt, P., Hong, S. C., Barlow, A., Janeway, C. A., Jr. (1991) Sequence analysis of peptides bound to MHC class II molecules. *Nature* **353,** 622–627.
7. Falk, K., Rötzschke, O., Stevanović, S., Jung, G., Rammensee, H. G. (1991) Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature* **351,** 290–296.
8. Dengjel, J., Schoor, O., Fischer, R., Reich, M., Kraus, M., Müller, M., Kreymborg, K., Altenberend, F., Brandenburg, J., Kalbacher, H., Brock, R., Driessen, C., Rammensee, H. G., Stevanovic, S. (2005) Autophagy promotes MHC class II presentation of peptides from intracellular source proteins. *Proc. Natl. Acad. Sci. USA* **102,** 7922–7927.
9. Suri, A., Vidavsky, I., van der Drift, K., Kanagawa, O., Gross, M. L., Unanue, E. R. (2002) In APCs, the autologous peptides selected by the diabetogenic I-Ag7 molecule are unique and determined by the amino acid changes in the P9 pocket. *J. Immunol.* **168,** 1235–1243.
10. Muntasell, A., Carrascal, M., Serradell, L., Veelen Pv, Pv., Verreck, F., Koning, F., Raposo, G., Abián, J., Jaraquemada, D. (2002) HLA-DR4 molecules in neuroendocrine epithelial cells associate to a heterogeneous repertoire of cytoplasmic and surface self peptides. *J. Immunol.* **169,** 5052–5060.
11. Muntasell, A., Carrascal, M., Alvarez, I., Serradell, L., van Veelen, P., Verreck, F. A., Koning, F., Abián, J., Jaraquemada, D. (2004) Dissection of the HLA-DR4 peptide repertoire in endocrine epithelial cells: strong influence of invariant chain and HLA-DM expression on the nature of ligands. *J. Immunol.* **173,** 1085–1093.
12. Costantino, C. M., Spooner, E., Ploegh, H. L., Hafler, D. A. (2012) Class II MHC self-antigen presentation in human B and T lymphocytes. *PLoS One* **7,** e29805.
13. Collado, J. A., Alvarez, I., Ciudad, M. T., Espinosa, G., Canals, F., Pujol-Borrell, R., Carrascal, M., Abián, J., Jaraquemada, D. (2013) Composition of the HLA-DR-associated human thymus peptidome. *Eur. J. Immunol.* **43,** 2273–2282.
14. Marrack, P., Ignatowicz, L., Kappler, J. W., Boymel, J., Freed, J. H. (1993) Comparison of peptides bound to spleen and thymus class II. *J. Exp. Med.* **178,** 2173–2183.
15. Alvarez, I., Collado, J. A., Colobran, R., Carrascal, M., Ciudad, M. T., Canals, F., James, E. A., Kwok, W. W., Gärtner, M., Kyewski, B., Pujol-Borrell, R., Jaraquemada, D. (2015) Central T cell tolerance: identification of tissue-restricted autoantigens in the thymus HLA-DR peptidome. *J. Autoimmun.* **60,** 12–19.
16. Bozzacco, L., Yu, H., Zebroski, H. A., Dengjel, J., Deng, H., Mojsov, S., Steinman, R. M. (2011) Mass spectrometry analysis and quantitation of peptides presented on the MHC II molecules of mouse spleen dendritic cells. *J. Proteome Res.* **10,** 5016–5030.
17. Dongre, A. R., Kovats, S., deRoos, P., McCormack, A. L., Nakagawa, T., Paharkova-Vatchkova, V., Eng, J., Caldwell, H., Yates III, J. R., Rudensky, A. Y. (2001) In vivo MHC class II presentation of cytosolic proteins revealed by rapid automated tandem mass spectrometry and functional analyses. *Eur. J. Immunol.* **31,** 1485–1494.
18. Clement, C.C., Becerra, A., Yin, L., Zolla, V., Huang, L., Merlin, S., Follenzi, A., Shaffer, S.A., Santambrogio, L., Stern, L.J. (2016) The dendritic cell MHC II peptidome derives from a variety of processing pathways and includes peptides with a broad spectrum of HLA-DM sensitivity. *J. Biol. Chem.* **291,** 5576–5595.
19. Muixí, L., Contreras, V., Collado, J. A., Alexandre, Y., Ballingall, K., Bonneau, M., Jaraquemada, D., Schwartz-Cornil, I. (2012) Unraveling features of the natural MHC class II peptidome of skin-migrated dendritic cells. *Int. Immunol.* **24,** 59–69.
20. Adamopoulou, E., Tenzer, S., Hillen, N., Klug, P., Rota, I. A., Tietz, S., Gebhardt, M., Stevanovic, S., Schild, H., Tolosa, E., Melms, A., Stoeckle, C. (2013) Exploring the MHC-peptide matrix of central tolerance in the human thymus. *Nat. Commun.* **4,** 2039.
21. Mommen, G.P., Marino, F., Meiring, H.D., Poelen, M.C., van Gaans-van den Brink, J.A., Mohammed, S., Heck, A.J., van Els, C.A. (2016) Sampling from the proteome to the HLA-DR ligandome proceeds via high specificity. *Mol. Cell. Proteomics* **15,** 1412–1423.
22. Röhn, T. A., Boes, M., Wolters, D., Spindeldreher, S., Müller, B., Langen, H., Ploegh, H., Vogt, A. B., Kropshofer, H. (2004) Upregulation of the CLIP self peptide on mature dendritic cells antagonizes T helper type 1 polarization. *Nat. Immunol.* **5,** 909–918.
23. Van Haren, S.D., Herczenik, E., ten Brinke, A., Mertens, K., Voorberg, J., Meijer, A.B. (2011) HLA-DR-presented peptide repertoires derived from human monocyte-derived dendritic cells pulsed with blood coagulation factor VIII. *Mol. Cell. Proteomics* **10,** M110.002246.
24. van Haren, S. D., Wroblewska, A., Herczenik, E., Kaijen, P. H., Ruminska, A., ten Brinke, A., Meijer, A. B., Voorberg, J. (2013) Limited promiscuity of HLA-DRB1 presented peptides derived of blood coagulation factor VIII. *PLoS One* **8,** e80239.
25. Sorvillo, N., van Haren, S. D., Kaijen, P. H., ten Brinke, A., Fijnheer, R., Meijer, A. B., Voorberg, J. (2013) Preferential HLA-DRB1*11-dependent presentation of CUB2-derived peptides by ADAMTS13-pulsed dendritic cells. *Blood* **121,** 3502–3510.
26. Sorvillo, N., Hartholt, R.B., Bloem, E., Sedek, M., Ten Brinke, A., van der Zwaan, C., van Alphen, F.P., Meijer, A.B., Voorberg, J. (2015) von Willebrand factor binds to the surface of dendritic cells and modulates peptide presentation of factor VIII. *Haematologica* **101,** 309–318.
27. ProPred. MHC class-II binding peptide prediction server. Available at: http://www.imtech.res.in/raghava/propred/. Accessed September 21, 2015.
28. Center for Biological Sequence Analysis. NetMHCIIpan 3.1 server. Available at: http://www.cbs.dtu.dk/services/NetMHCIIpan/. Accessed September 21, 2015.
29. Laboratori de Proteomica. Laia motifs. Available at: http://proteomica.uab.cat/index.php?option=com_content&view=article&id=76. Accessed September 21, 2015.
30. Alvarez, I., Collado, J., Daura, X., Colomé, N., Rodríguez-García, M., Gallart, T., Canals, F., Jaraquemada, D. (2008) The rheumatoid arthritis-associated allele HLA-DR10 (DRB1*1001) shares part of its repertoire with HLA-DR1 (DRB1*0101) and HLA-DR4 (DRB*0401). *Arthritis Rheum.* **58,** 1630–1639.
31. Vogt, A. B., Kropshofer, H., Kalbacher, H., Kalbus, M., Rammensee, H. G., Coligan, J. E., Martin, R. (1994) Ligand motifs of HLA-DRB5*0101 and DRB1*1501 molecules delineated from self-peptides. *J. Immunol.* **153,** 1665–1673.
32. Suri, A., Lovitch, S. B., Unanue, E. R. (2006) The wide diversity and complexity of peptides bound to class II MHC molecules. *Curr. Opin. Immunol.* **18,** 70–77.
33. Gene Ontology Consortium. Gene ontology database. Available at: www.geneontology.org. Accessed September 23, 2015.
34. Crotzer, V. L., Blum, J. S. (2010) Autophagy and adaptive immunity. *Immunology* **131,** 9–17.
35. Holland, C. J., Cole, D. K., Godkin, A. (2013) Re-directing CD4(+) T cell responses with the flanking residues of MHC class II-bound peptides: the core is not enough. *Front. Immunol.* **4,** 172.
36. Painter, C. A., Stern, L. J. (2012) Conformational variation in structures of classical and non-classical MHCII proteins and functional implications. *Immunol. Rev.* **250,** 144–157.
37. Burster, T., Boehm, B. O. (2010) Processing and presentation of (pro)-insulin in the MHC class II pathway: the generation of antigen-based immunomodulators in the context of type 1 diabetes mellitus. *Diabetes Metab. Res. Rev.* **26,** 227–238.
38. Wellcome Trust Sanger Institute. MEROPS: the peptidase database. Available at: https://merops.sanger.ac.uk/. Accessed April 5, 2016.
39. Burnam Institute for Medical Research. PMAP-CutDB proteolytic event database. Available at: cutdb.burnham.org. Accesssed April 5, 2016.
40. Pereira, S. R., Faça, V. M., Gomes, G. G., Chammas, R., Fontes, A. M., Covas, D. T., Greene, L. J. (2005) Changes in the proteomic profile during differentiation and maturation of human monocyte-derived dendritic cells stimulated with granulocyte macrophage colony stimulating factor/interleukin-4 and lipopolysaccharide. *Proteomics* **5,** 1186–1198.
41. Collado, J. A., Guitart, C., Ciudad, M. T., Alvarez, I., Jaraquemada, D. (2013) The repertoires of peptides presented by MHC-II in the thymus and in peripheral tissue: a clue for autoimmunity? *Front. Immunol.* **4,** 442.

42. Nelson, C. A., Petzold, S. J., Unanue, E. R. (1993) Identification of two distinct properties of class II major histocompatibility complex-associated peptides. *Proc. Natl. Acad. Sci. USA* **90,** 1227–1231.
43. Arnold, P. Y., La Gruta, N. L., Miller, T., Vignali, K. M., Adams, P. S., Woodland, D. L., Vignali, D. A. (2002) The majority of immunogenic epitopes generate CD4+ T cells that are dependent on MHC class II-bound peptide-flanking residues. *J. Immunol.* **169,** 739–749.
44. O'Brien, C., Flower, D. R., Feighery, C. (2008) Peptide length significantly influences in vitro affinity for MHC class II molecules. *Immunome Res.* **4,** 6.
45. Anders, A. K., Call, M. J., Schulze, M. S., Fowler, K. D., Schubert, D. A., Seth, N. P., Sundberg, E. J., Wucherpfennig, K. W. (2011) HLA-DM captures partially empty HLA-DR molecules for catalyzed removal of peptide. *Nat. Immunol.* **12,** 54–61.
46. Zavala-Ruiz, Z., Strug, I., Walker, B. D., Norris, P. J., Stern, L. J. (2004) A hairpin turn in a class II MHC-bound peptide orients residues outside the binding groove for T cell recognition. *Proc. Natl. Acad. Sci. USA* **101,** 13279–13284.
47. Cole, D. K., Gallagher, K., Lemercier, B., Holland, C. J., Junaid, S., Hindley, J. P., Wynn, K. K., Gostick, E., Sewell, A. K., Gallimore, A. M., Ladell, K., Price, D. A., Gougeon, M. L., Godkin, A. (2012) Modification of the carboxy-terminal flanking region of a universal influenza epitope alters CD4$^+$ T-cell repertoire selection. *Nat. Commun.* **3,** 665.
48. Lippolis, J. D., White, F. M., Marto, J. A., Luckey, C. J., Bullock, T. N., Shabanowitz, J., Hunt, D. F., Engelhard, V. H. (2002) Analysis of MHC class II antigen processing by quantitation of peptides that constitute nested sets. *J. Immunol.* **169,** 5089–5097.
49. Godkin, A. J., Smith, K. J., Willis, A., Tejada-Simon, M. V., Zhang, J., Elliott, T., Hill, A. V. S. (2001) Naturally processed HLA class II peptides reveal highly conserved immunogenic flanking region sequence preferences that reflect antigen processing rather than peptide-MHC interactions. *J. Immunol.* **166,** 6720–6727.
50. Vigneron, N., Van den Eynde, B. J. (2014) Proteasome subtypes and regulators in the processing of antigenic peptides presented by class I molecules of the major histocompatibility complex. *Biomolecules* **4,** 994–1025.
51. Kim, A., Sadegh-Nasseri, S. (2015) Determinants of immunodominance for CD4 T cells. *Curr. Opin. Immunol.* **34,** 9–15.
52. Watts, C., Matthews, S. P., Mazzeo, D., Manoury, B., Moss, C. X. (2005) Asparaginyl endopeptidase: case history of a class II MHC compartment protease. *Immunol. Rev.* **207,** 218–228.
53. Oliveira, C. C., Querido, B., Sluijter, M., de Groot, A. F., van der Zee, R., Rabelink, M. J., Hoeben, R. C., Ossendorp, F., van der Burg, S. H., van Hall, T. (2013) New role of signal peptide peptidase to liberate C-terminal peptides for MHC class I presentation. *J. Immunol.* **191,** 4020–4028.
54. Kropshofer, H., Max, H., Halder, T., Kalbus, M., Muller, C. A., Kalbacher, H. (1993) Self-peptides from four HLA-DR alleles share hydrophobic anchor residues near the NH2-terminal including proline as a stop signal for trimming. *J. Immunol.* **151,** 4732–4742.
55. Max, H., Halder, T., Kropshofer, H., Kalbus, M., Müller, C. A., Kalbacher, H. (1993) Characterization of peptides bound to extracellular and intracellular HLA-DR1 molecules. *Hum. Immunol.* **38,** 193–200.

KEY WORDS:
Ag processing · Ag presentation · MHC class II · autologous peptides · mass spectrometry