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Membranal and blood-soluble HLA class II peptidome analyses using data-dependent and independent acquisition

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Abstract

The interaction between HLA class II peptide complexes on antigen-presenting cells and $CD4^+$ T cells is of fundamental importance for anti-cancer and anti-pathogen immunity as well as for the maintenance of immunological tolerance. To study $CD4^+$ T cell reactivities, detailed knowledge of the presented peptides is necessary. In recent years, dramatic advances in the characterization of membranal and soluble HLA class I peptidomes could be observed. However, the same is not true for HLA class II peptidomes, where only few studies identify more than hundred peptides. Here we describe a mass spectrometry-based workflow for the characterization of membranal and soluble HLA class II DR and DQ peptidomes. Using this workflow, we identified a total of 8595 and 3727 HLA class II peptides from Maver-1 and DOHH2 cells, respectively. Based on this data, a motif-based binding predictor was developed and compared to NetMHCIIpan 3.1. We then applied the workflow to human plasma, resulting in the identification of between 34 and 152 HLA-DR and between 100 and 180 HLA-DQ peptides, respectively. Finally, we implemented a data-independent acquisition workflow to increase reproducibility and sensitivity of HLA class II peptidome characterizations.

Keywords

Biomarker; DIA; HLA class II; HLA peptidomics; immunopeptidomics

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1 Introduction

Human leukocyte antigen (HLA) class II complexes present peptides on the surface of professional antigen presenting cells (APC). Displayed peptides originate mainly from endosomal degradation of endogenous and exogenous proteins and have a variable length of around 15 amino acids [1]. The presented HLA-peptide complexes can maintain tolerance or activate $CD4^+$ T cells, depending on secondary signals. Activated $CD4^+$ T cells can detect pathologic events, such as malignant transformation or infection with pathogens, and induce an appropriate immune response [2, 3]. $CD4^+$ T cells further maintain immunological tolerance by differentiating into regulatory T cells [2]. If the fine balance between pro- and anti-inflammatory signals is broken, autoimmunity can develop [4]. Besides membrane-bound HLA class II complexes, a soluble form of the HLA class II complexes (sHLA-II) has been detected in serum as well as in various other body fluids [5, 6]. The function of these sHLA-II complexes remains to be determined, but a role in the induction and maintenance of immunological tolerance has been proposed [7, 8].

In recent years, it was also observed that $CD4^+$ T cells can play an important role in cancer immunotherapy [9]. Traditionally, $CD8^+$ T cells were assumed to be responsible for rejecting established tumors, and were therefore selected as the major target for vaccination strategies. However, innovative strategies are required to translate the promising preclinical observations into clinical success [10]. In mouse models, induction of $CD4^+$ T cell responses has recently been suggested to improve therapeutic efficacy [11]. Similarly, two clinical studies evaluating personalized neo-epitope vaccination strategies in patients with melanoma observed a pronounced induction of an immunological response only after stimulation of $CD4^+$ T cells [12, 13]. Both groups recognized that currently the development of vaccines inducing $CD4^+$ T cell responses is prevented by the fact that HLA class II peptide binding prediction is not sufficiently established. One possibility to improve the peptide binding prediction is the definition of HLA-specific motifs in high resolution. However, for HLA class II, only very few studies demonstrate the confident identification of hundreds of HLA class II peptides [14–18]. As a consequence, only very few HLA class II-specific motifs have been characterized sufficiently to allow peptide binding prediction [19].

In this work, we investigated a workflow for the purification and identification of HLA class II DR and DQ peptides. We established and tested the workflow using Maver-1 and DOHH2 cells resulting in the identification of 6075 DR and 2577 DQ peptides for Maver-1 and 3238 DR and 540 DQ peptides for DOHH2 cells, respectively. Based on the identified peptides, we generated a motif-based binding predictor and compared its performance to NetMHCIIpan 3.1. We then applied the workflow to the purification and identification of sHLA-II peptides from human plasma resulting in the identification of between 34 and 152 HLA-DR and 100 and 180 HLA-DQ peptides, respectively. In an attempt to increase sensitivity and reproducibility of HLA class II peptidome characterizations, we established a data-independent acquisition (DIA) workflow, which we apply to the analysis of HLA class II peptidomes from cell lines.

2 Materials and Methods

Cell lines and Antibodies

The human mantle cell lymphoma cell line MAVER-1 was a kind gift of Prof Alberto Zamo [20]. The human follicular lymphoma cell line DOHH-2 was obtained from the DSMZ, (Braunschweig). Cell lines were cultivated in RPMI 1640 medium (Gibco) with 10% heat-inactivated FCS (Gibco). HLA typing was performed by the MVZ (Martinsried, Germany) [Supporting Information Table 1]. The L243 hybridoma cell line (HB-55) and the W6/32 hybridoma cell line (HB-95) were obtained from ATCC, the SPVL3 hybridoma cell line was a kind gift of Prof Dr Hergen Spits. Hybridoma cells were cultivated in CD Hybridoma medium (Gibco) with 2 mM glutamine (Ultraglutamine I, Lonza), at 37°C and 5% CO2. The L243 [21–23], SPVL3 [24–29] and W6/32 antibodies were purified from the respective hybridoma supernatants using Protein-A Sepharose and subsequently coupled to AminoLink Plus Coupling Resin (Thermo Fisher Scientific) following the manufacturer`s instructions.

Cell lysis and human plasma

Cells were lysed as previously described [30]. Plasma from donors was generated by drawing blood with EDTA tubes (S-Monovette 9 ml K3E, Sarstedt). Tubes were readily inverted, incubated for 30 min at RT and centrifuged at 2’000g for 10 min. Plasma was transferred into new vials, snap-frozen and stored at -80°C. After thawing, plasma was cleared by centrifugation at 3’345g for 5 min, 4°C prior to HLA purification. All donors were from the Department of Nephrology (Tenon Hospital Paris, France) and provided written informed consent for usage of plasma and clinical data for scientific purposes. Donors one and two were healthy volunteers, donors three and four were patients suffering from the rare kidney disease membranous nephropathy. This project was approved by the IRB/Ethics committee CPP Ile-de-France IV, Hôpital Saint-Louis, Paris (ethic vote 2013/03/14).

Volunteers were HLA typed by NGS technologies resulting in an unambiguous annotation of the A, B, C, DR and DQ alleles [Supporting Information Table 1].

Affinity purification of HLA complexes and enrichment of HLA-bound peptides from cell lines and plasma

HLA class II complexes were purified from lysate or 2.5-3 ml of plasma using L243 antibody-coupled resin for HLA class II DR and SPVL3 antibody-coupled resin for HLA class II DQ complexes. Resin was incubated for 2 h at 4°C, then washed once with 10 ml of lysis buffer, buffer A (150 mM NaCl, 20 mM Tris, pH 7.4), buffer B (400 mM NaCl, 20 mM Tris, pH 7.4), buffer A again and buffer C (20 mM Tris, pH 8.0). Peptide-HLA complexes were eluted with 10% acetic acid. Peptides were separated from protein by elution from C18 Macro SpinColums (Harvard Apparatus) with 25% ACN, 0.1% TFA, and subsequently dried and stored at -20°C. HLA class I complexes were purified from plasma as described previously [31].

DDA analysis of HLA peptides by UHPLC MS

Purified dried HLA class II peptides were resuspended in 3% ACN, 0.1% formic acid and iRT peptides (Biognosys, Schlieren) were added to all of the samples. Peptides were analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) using a Q Exactive Mass Spectrometer fitted with an EASY-nLC 1000 (both Thermo Fisher Scientific). Peptides were resolved with an Acclaim PepMap RSLC C18, 50 μm x 150 mm, 2 μm analytical column (Thermo Fisher Scientific) at a flow rate of 0.3 μL/min by running a linear gradient from 0% to 30% ACN over 120 min. All buffers contained 0.1% formic acid. MS spectra were recorded in full ion scan mode from 380–2'000 m/z, with a resolution of 70'000 and a maximum injection time of 80 ms. MS/MS were recorded at a resolution of 17'500 and a maximum injection time of 240 ms. The ten most intense masses with charges of two to five were selected for HCD fragmentation. Dynamic exclusion was set to 10 seconds.

Resulting spectra were processed and analyzed using the Proteome Discoverer software (Thermo Fisher Scientific, Version 1.4.1.14). MS/MS data was searched against a database consisting of the human (93'250 entries) and the bovine (24'339 entries) reference proteomes downloaded from the UniProt homepage on the 7th of July 2017, spiked with the 11 iRT peptide sequences from the iRT kit. The following analysis settings were used with SEQUEST: i) No-Enzyme (Unspecific), ii) precursor mass tolerance 7 ppm, iii) fragment mass tolerance 0.02 Da, iv) one variable modification (oxidation of methionine). False discovery rates were calculated with the Percolator plug-in and filtered at 1% FDR. HLA class I peptides were analyzed as described [31].

Gibbs clustering of HLA class II peptides

HLA peptides were analyzed using the GibbsCluster-2.0 Server [32]. HLA class II peptides with a length of at least 9 amino acids were clustered using the predefined "MHC class II ligands" parameters.

Ovalbumin feeding of Maver-1 cells

Sterile-filtered Ovalbumin stock solution (10 mg/ml Ovalbumin in PBS) was added to Maver-1 cells 48h prior to cell lysis and again 6h before lysis at a final concentration of 0.16mg/ml medium. Maver-1 cells were lysed and HLA class II peptides were isolated and analyzed as described above.

HLA class I binding prediction of identified peptides

Peptides with a length between 8 and 11 amino acids were subjected to HLA class I binding prediction analysis using NetMHCpan 3.0 [33]. Each peptide was assigned the minimal predicted peptide rank (%). Peptides were annotated as being predicted to bind if the recalculated rank was below 2 % for at least one of the alleles of the cell line.

Matrix-based scoring of peptide sequences and peptide-HLA class II binding prediction

Data from Ooi et al. [18] and from Clement et al. [17] were the basis for the calculation of position-specific scoring matrices (PSSM). Peptides identified from mono-allelic cells, or

from cells expressing DRB1*15:01 and DRB5*01:01 were submitted to GibbsCluster-2.0 Server [32] analysis using the standard parameters for MHC class II including preference for hydrophobic AAs at P1. Core sequences identified from the clustering analysis of the DRB1*01:01, DRB1*15:01 and DRB5*01:01 clusters were submitted to Seq2Logo 2.0 server [34], using the following parameters: Logo type: Kullback-Leibler, Clustering method: Hobohm1, Threshold for clustering: 0.63, Weight on prior: 200, Information content: Bits, in the advanced settings, Blossum62 matrix and respective background frequencies were enabled. The position-specific scoring matrix (PSSM) was downloaded after calculation of the sequence logo. Scoring and binding prediction was performed essentially as described [19], applying a sliding window of nine amino acids to each peptide sequence. Core regions (i.e. the region binding to the cognate HLA class II allele through anchor residues) were defined by the region with the highest score. A ROC curve analysis of peptides identified from cell lines positive for a given allele and of all 15mers calculated for the human reference proteome (93’250 protein entries) downloaded from the UniProt homepage on 07th July 2017 was used to calculate score cutoff values defining predicted binding.

Prediction of peptide-HLA class II binding with the NetMHCIIpan 3.1 server [33] was performed keeping all standard parameters, selecting the HLA class II allele and submitting peptide sequences with at least nine amino acids. The threshold for binding was defined as a rank below 10%.

Spectral library generation

Libraries were generated essentially as described [35] using SpectraST (Version 5.0.0) [36]. Libraries were based on the peptides identified from SEQUEST with a 1% FDR performing retention time alignment with Spectrast2irt_alignment.py. The consensus spectrast.splib file was converted to a transition list using spectrast2tsv.py with the following settings: spectrast2tsv.py -l 250,2000 -s b,y -x 1,2 -o 3 -n 6 -g -17.03,-18.01 -p 0.05 -d -e -w windows.txt -k openswath –a output.csv input.sptxt.

DIA analysis was performed as in [35] with small modifications: The DIA method consisted of a survey scan from 380-2000 m/z at a resolution of 70’000. Equally spaced DIA windows of 30 m/z [Supporting Information Table 2] were recorded at a resolution of 35’000 in profile mode with fixed first mass set to 200 m/z and an NCE of 27. Raw files were centroided, converted to the mz5 format using msconvert (ProteoWizzard) and analyzed by Skyline (version 3.6.0.10162) [37] [Supporting Information Table 3]. Transition lists were pasted into Skyline, a retention time calibrator based on the iRT kit was created and decoys were added (decoy generation method: Reverse Sequence). Samples were imported into Skyline and peaks were filtered for an mProphet q-value below 0.01.

3 Results

HLA class II peptidomes of the human mantle cell lymphoma cell line Maver-1 and the human follicular lymphoma cell line DOHH2 were characterized following affinity purification of DR or DQ complexes with anti-HLA class II DR antibody L243 or anti-HLA class II DQ antibody SPVL3 as outlined in Figure 1A. Both B cell lymphoma cell lines were

HLA typed resulting in an unambiguous annotation of their DR and DQ alleles [Supporting Information Table 1]. Purified peptides were analyzed on a Q Exactive mass spectrometer, applying a 1% FDR on peptide identifications. HLA class II DR purifications resulted in the identification of a total of 6075 peptides from Maver-1 and 3238 peptides from DOHH2 cells [Supporting Information Table 4 a and b for a list of all peptide identifications]. The peptide length distributions of the DR peptides for both cell lines centered around 15 amino acids with the majority of peptides ranging between 12 and 22 amino acids [Figure 1B], consistent with previous reports on HLA class II peptide length [14, 15]. HLA class II DQ purifications resulted in the identification of a total of 2577 peptides from Maver-1 and 540 peptides from DOHH2 cells [Supporting Information Table 4 c and d for a list of all peptide identifications] with a length distribution comparable to DR peptides [Figure 1C].

We then evaluated if it was possible to extract the HLA-specific motifs for DR1*01:01, DR1*13:01, and DR3*02:02 from the Maver-1 DR peptidome. Gibbs cluster reported HLA-specific motifs only for two of the three DR alleles [see Supporting Information Figure 1]. Similarly, NetMHCIIpan 3.1 was able to identify putative DRB1*01:01-derived peptides, but did not report sequence logos with defined anchor residues at P4, P6, or P9 for DRB1*13:01 and DRB3*02:02 [see Supporting Information Figure 1]. Only after subtracting all putative DRB1*01:01 peptides, Gibbs cluster analysis of remaining peptides resulted in two distinct motifs, thereby finally revealing the three HLA-DR-specific motifs of Maver-1 in high resolution [Figure 1D and Supporting Information Figure 1]. Comparison of the data with the SYFPEITHI database [38] confirmed the annotation to respective alleles. Gibbs cluster also only revealed two of the three HLA DR-specific motifs of DOHH2 cells, while NetMHCIIpan 3.1 reported comparable core sequences to bind to all three alleles [see Supporting Information Figure 2]. Based on the DR1, DR15 and DR51-specific motifs obtained from the data reported by Ooi and colleagues [18], it was possible to assign the identified sequences to one of the three alleles, respectively [Figure 1E and Supporting Information Figure 2]. Gibbs cluster results for DQ revealed one and two clusters for Maver-1 [Figure 1F] and DOHH2 [Figure 1G], respectively.

In the past, we found high-resolution murine MHC class II-specific motifs to be very useful in predicting which regions of a protein can be presented by APCs [19]. To test if this was also the case for the human HLA class II-specific motifs, we were feeding ovalbumin to Maver-1 cells prior to analysis of the HLA DR peptidome. In total 21 peptide sequences clustering around three cores were identified [Supporting Information Table 5]. The three identified core sequences had further the highest and second highest score of all possible ovalbumin 9mer sequences. In contrast, NetMHCIIpan 3.1 predicted the binding of only one of the three cores to either DRB1*01:01 and DRB1*13:01 and reported two, five, and ten peptide sequences of ovalbumin to have a better rank score as compared to the identified core sequences.

We further investigated on the possibility to elute peptides from sHLA-II complexes present in circulation [6], in analogy to our previous report on sHLA-class I peptides isolated from serum and plasma [30, 31]. Based on the low blood concentration of HLA class II complexes, we expected to be able to sequence very few peptides. Therefore, we first validated the compatibility of the obtained plasma preparations from four donors by

purifying their sHLA-I complexes [Supporting Information Table 1 for the HLA typing of the four donors]. Reassuringly, results were comparable with our previous findings, resulting in between 702 and 2084 peptide sequences from 2.5-3 ml of plasma [Figure 2A]. Moreover, the quality of HLA class I preparations was high, with between 87.9 and 96.6% of all identified 8-11mers being predicted to bind the respective HLA alleles of the donors by NetMHCpan 3.0 [Figure 2B].

From 2.5-3 ml of plasma of the four donors, 34, 152, 91, and 77 putative HLA DR-derived peptide sequences were identified [Supporting Information Table 6 for a complete list of peptide identifications]. Peptide length distributions presented in Figure 2C demonstrate enrichment of longer peptide sequences as compared to class I. After removing obvious contaminants such as Keratin, Fibrinogen and other commonly identified sequences, we found 26 high-quality peptides from 14 proteins [Table 1]. Of these, five were previously identified from human thymus samples by Collado and colleagues [39], 8 were identified from monocyte-derived dendritic cells by Ciudad and colleagues [40], seven were identified from Maver-1 cells, and three from DOHH2 cells. Further, HLA DR alleles of the donors were typically in line with the sample from which the same peptide sequence was previously identified [Table 1].

After HLA DQ purification, 180, 100, 154, and 137 peptide sequences could be identified from plasma of the four donors, respectively [Supporting Information Table 7 for a complete list of peptide identifications]. Peptide length distributions presented in Figure 2D demonstrate enrichment of peptides with an average length of around 15 amino acids. After removing most obvious contaminants, 34 peptide sequences from 16 proteins remained [Table 2] of which 16 were previously identified by Bergseng and colleagues [14], indicating that they can be presented on DQ complexes.

To increase the sensitivity and reproducibility of HLA class II peptidome analyses, an attractive strategy might be the application of data-independent acquisition (DIA) workflows as this significantly increased the sensitivity and reproducibility of HLA class I peptidome analyses [35]. We therefore designed a DIA setup [Supporting Information Table 2] with 21 windows between 380 and 1010 m/z with a fixed size of 30 Da, the area in which most peptides with charge state 2, 3, 4 and 5 were present [Figure 3A]. Applying DR- and DQ-specific spectral libraries for Maver-1 and DOHH2 cells to DIA analyses, more than 90% of the library peptides could be recovered with Skyline [Figure 3B and Supporting Information Table 3 for Skyline settings]. The DIA analyses resulted in the identification of a total of 5939 DR peptides and 2505 DQ peptides for Maver-1 cells with overlaps between triplicates of 88.52% and 94.85%, respectively [Figure 3C left panels]. For the DOHH2 cell line, 3157 DR peptides and 481 DQ peptides were identified, with overlaps of 93.19% and 91.06% between triplicates [Figure 3C right panels]. We then evaluated on the sensitivity of the workflow by purifying HLA class II DR peptides from Maver-1 lysate corresponding to 100, 25, 10, 5, and 1 million cells. The DIA workflow increased the identification rate in all samples, with the strongest effect observed for samples with lower input [Figure 3D]. Using the DIA workflow, up to an average of 1552 peptides could be identified from 1 million cells, more than 4 fold the number of identifications as from DDA samples.

4 Discussion

The definition of HLA-specific motifs in high resolution is important as they facilitate evaluation and prediction if a given peptide can be presented by certain HLA complexes. Such strategies have been implemented for the design of cancer vaccines [11–13] and for prediction of immunogenicity of biologic drugs [41]. For HLA class I, large databases of HLA-specific motifs have been collected and are publicly available [42, 43]. In contrast, few HLA class II-specific motifs have been defined in high resolution, including DRB1*01:01, DRB1*15:01, DRB1*08:01, DRB1*10:01, DRB5*01:01 [17, 18, 44, 45]. One difficulty relates to the nature of the class II peptides, which feature a central core and overlapping ends. This obviously complicates deconvolution of binding motifs if more than two alleles are present, especially when motifs are similar (such as DRB1*01:01 and DRB5*01:01). This is exemplified by the fact that Gibbs cluster failed to reveal the three allele-specific motifs expected for Maver-1 and DOHH2 cells, while it usually separates three to five distinct clusters for HLA class I data [30]. This is even more problematic for HLA DQ, where the only available dataset with acceptable number of peptides was only recently presented [14]. The DQ-specific motifs reported so far are however not very specific, and do not feature the typical setup of anchor residues at position P1, P4, P6, P9, typically observed for DR alleles, thereby raising doubt on their quality and validity. If Gibbs cluster was able to identify the real motifs from our HLA DQ preparations is very difficult to evaluate due to the scarcity of HLA-DQ data. Our cell lines did not share any of the HLA-DQB alleles with the ones studied by Bergseng and colleagues [14], while, the reported motifs are comparable as such that they are less defined in terms of their anchor residues. In the future, it will be important to study more mono-allelic cell lines, reanalyze existing data with specialized bioinformatics strategies and evaluate the influence of different DQB1-DQA1 combinations, as it is believed that DQ cis- and trans-pairing can occur [46].

The pipeline for the identification of thousands of high confidence peptides eluted from HLA DR and DQ complexes was subsequently applied to the study of blood-soluble HLA-II complexes resulting in the detection of between 34 and 180 sequences. Due to the low concentration of class II complexes as compared to class I complexes, the low number of identifications is not surprising. However, we believe that we indeed were able to detect eluted peptides, as some of the peptide sequences have been previously detected from cultured cells and from thymus lysates (Table 1 and Table 2). In the future, with more sensitive mass spectrometers it is very well possible that higher numbers of peptides can be identified which might serve as biomarkers for inflammatory diseases such as arthritis, multiple sclerosis or inflammatory bowel disease.

Finally, we implemented DIA of HLA class II peptidomes following initial work on HLA class I. We could demonstrate that also for HLA class II, DIA methods increase the sensitivity and reproducibility of the resulting peptidomes significantly. DIA strategies will allow to identify and quantify disease relevant antigen in scarce clinical samples such as bronchoalveolar lavage fluid [47], or similar biopsy material [39, 40], with the goal to develop tolerance inducing therapeutic strategies.

In summary, we isolated and identified human HLA class II DR and DQ peptides leading to the precise definition of HLA-specific motifs allowing the prediction of peptide presentation on respective alleles. We further investigated on the presence of sHLA-II complexes in blood and could for the first time demonstrate that the complexes are still associated with peptide ligands. We finally established a DIA methodology resulting in the reliable and sensitive definition of HLA class II peptidomes, probably applicable to a range of biological questions. Given the importance of HLA class II peptides in modulating immune responses, e.g. by cancer vaccines [11], and in autoimmune condition [7], it is imperative to expand current efforts to the definition of additional HLA class II-specific motifs, allowing the development of refined binding prediction tools and of personalized therapeutic strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations

HLA	human leukocyte antigen
DDA	data-dependent acquisition
DIA	data-independent acquisition

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Significance of the study

Peptides displayed on HLA class II complexes on the surface of antigen presenting cells play a central role in the activation of $CD4^+$ T cells and as such are of fundamental importance for anti-cancer and anti-pathogen immunity as well as for the maintenance of immunological tolerance. Recent studies evaluating cancer vaccination strategies observed a pronounced response only upon stimulation of $CD4^+$ T cells. However, development of novel personalized anti-cancer vaccines targeting $CD4^+$ T cells is hampered by the fact that HLA class II peptide binding prediction is not sufficiently established. In this study, we present strategies for the identification of HLA class II peptides from cell lines for the development of an HLA allele-specific motif-based binding predictor. Such tools may facilitate design of more efficacious cancer vaccines. We further investigate on peptides presented on blood-soluble HLA class II complexes (sHLA-II). While their function is believed to contribute to immunological tolerance, it was unknown if they are still bound to cognate antigen, or if the peptide is rapidly lost. Identifying sHLA-II peptides may allow to study their function and may allow to identify biomarkers of HLA class II-associated diseases such as multiple sclerosis or rheumatoid arthritis.

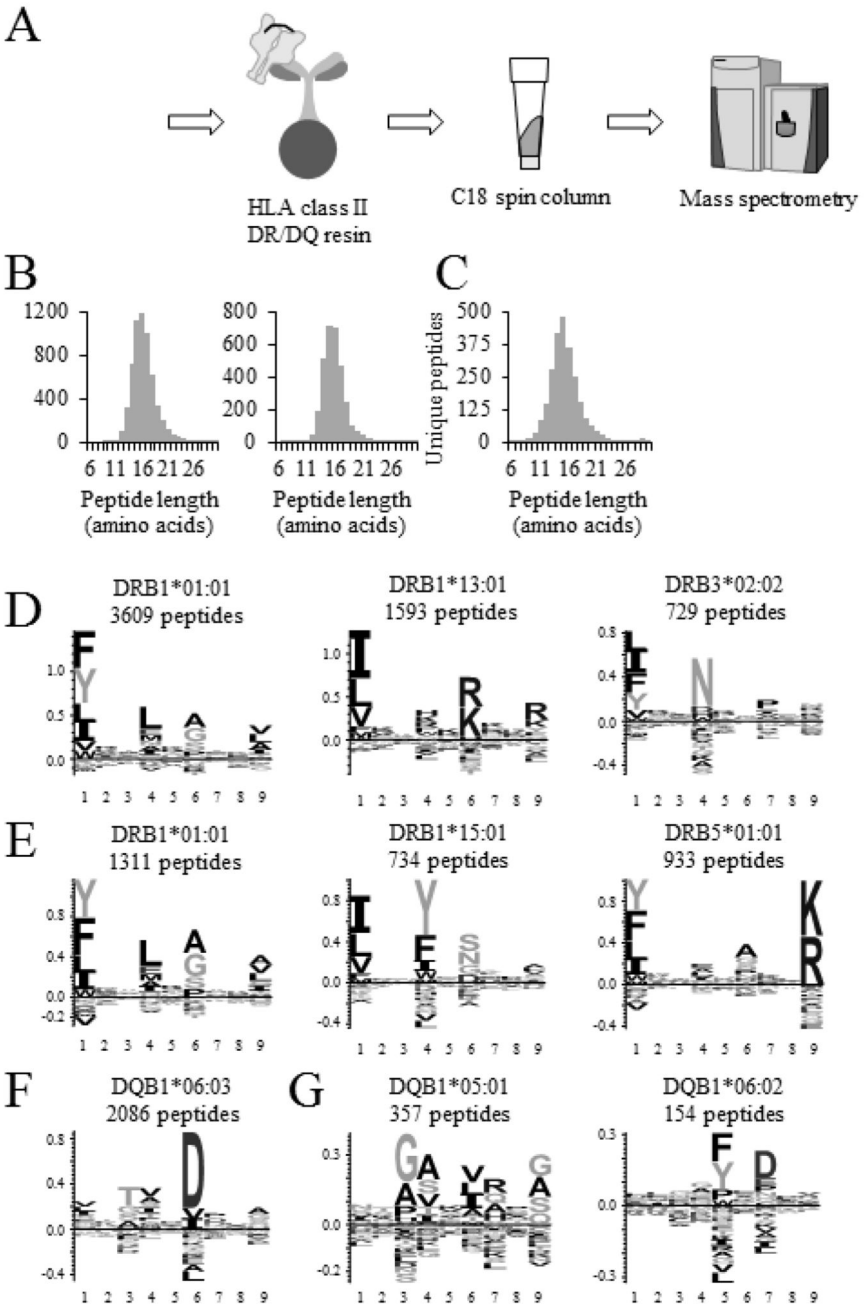

Figure 1. HLA class II DR and DQ peptidome analysis of Maver-1 and DOHH2 cells.

(A) Schematic representation of the purification of HLA class II peptides. HLA class II complexes are purified from cell lysates and plasma using either L243-conjugated resin (HLA DR) or SPVL3-conjugated resin (HLA DQ). After acid elution of HLA class II complexes, peptides are separated from proteins by stepwise elution from C18 resin and analyzed by LC-MS/MS. **(B, C)** Length distribution of identified peptides from HLA class II purifications. The number of peptides between 6 and 30 amino acids is plotted in relation to their length. Length distributions for HLA class II DR peptides **(B)** and for HLA class II DQ

peptides **(C)** are shown. Left panel Maver-1, right panel DOHH2, respectively (**D-G**) Definition of HLA-specific motifs from HLA class II DR and DQ purifications. HLA class II-specific motifs were created from all unique peptide sequences with a minimum length of 9 amino acids isolated from Maver-1 (**D**, **F**) or DOHH2 cells (**E**, **G**) using the GibbsCluster-2.0 Server.

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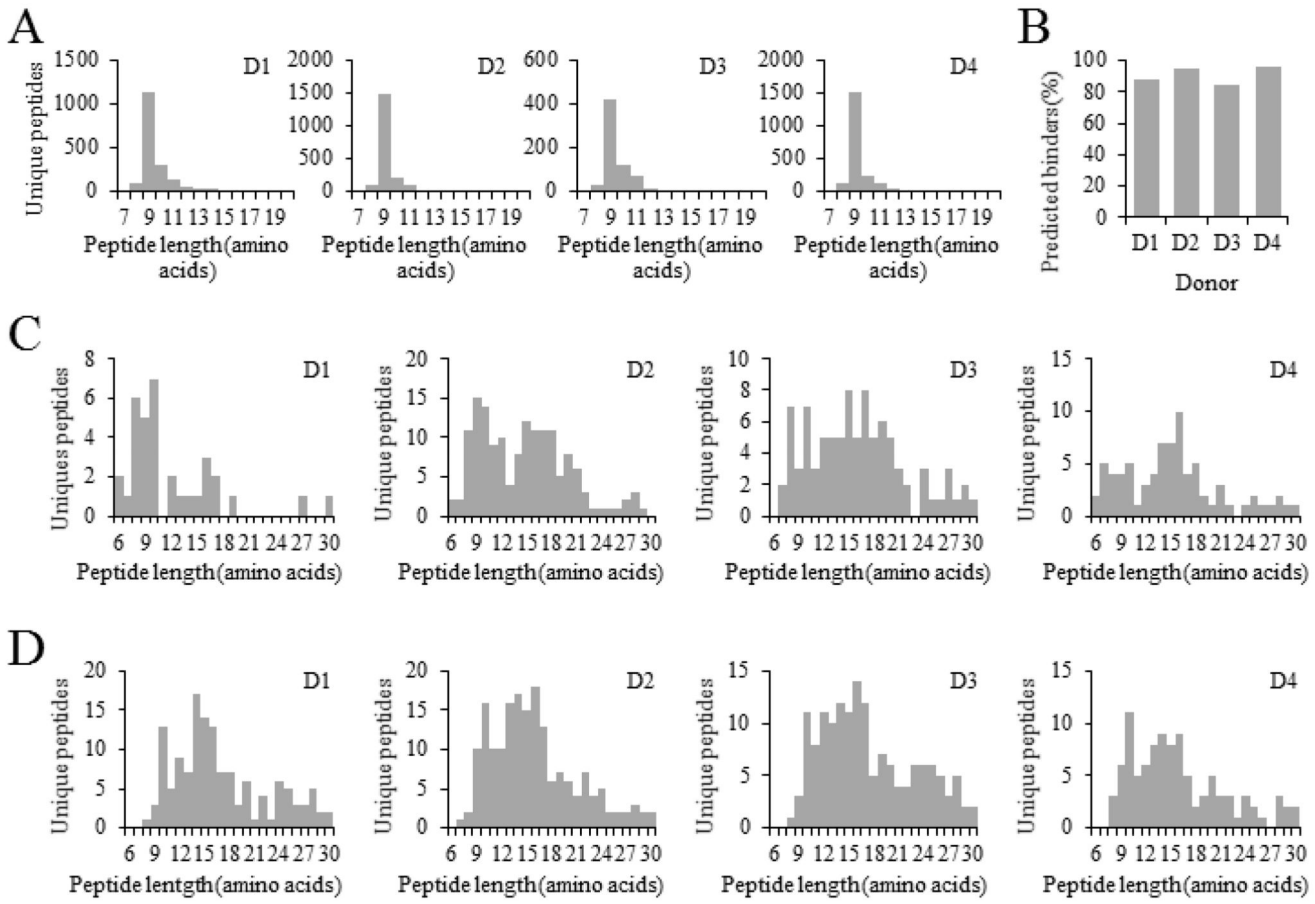

Figure 2. Analysis of the soluble HLA class I and class II complexes from plasma.

(**A**) Length distribution of sHLA-I peptides isolated from 4 donors (D1 to D4). The number of peptides between 7 and 20 amino acids is plotted in relation to their length. (**B**) NetMHCpan 3.0 binding prediction of 8 to 11-mers identified from plasma. Peptides were annotated as being predicted to bind if the rank received from NetMHCpan was below 2% for at least one of the alleles of the respective donor. (**C, D**) Length distribution of sHLA-II peptides isolated from plasma. sHLA-II DR peptides (**C**) or sHLA-II DQ peptides (**D**) were isolated from 2.5-3 mL of plasma from four donors (D1 to D4). The number of peptides between 6 and 30 amino acids is plotted in relation to their length.

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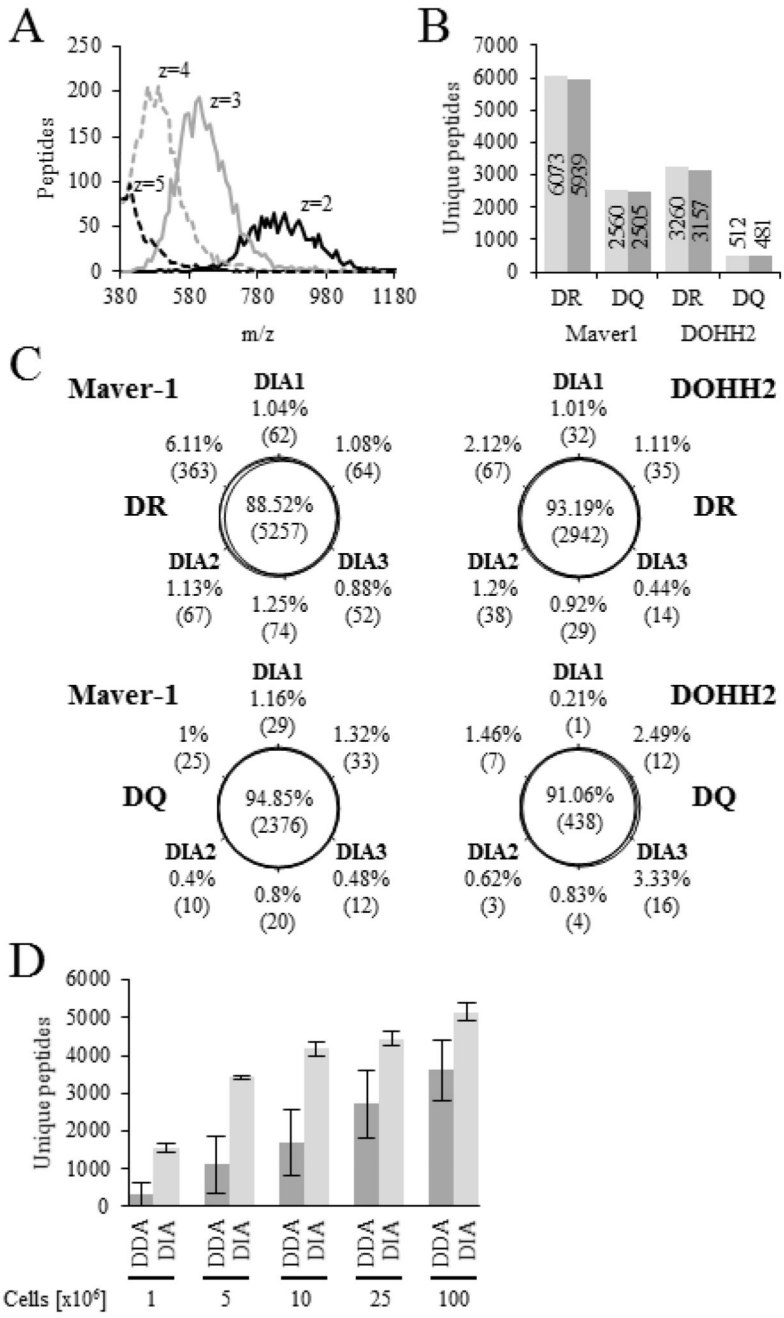

Figure 3. DIA analysis of HLA DR and DQ peptidomes of Maver-1 and DOHH2 cells.

(**A**) Precursor mass distribution from a triplicate DDA analysis of the Maver-1 HLA DR peptidome. The m/z distributions of all peptides with charge state 2 (z=2, solid black), 3 (z=3, solid grey), 4 (z=4, dotted grey) and 5 (z=5, dotted black) were plotted. (**B**) Library generation and DIA analysis of Maver-1 and DOHH2 cells. Spectral libraries were created using SpectraST based on SEQUEST results filtered to 1% FDR with Percolator from triplicate DR and DQ DDA analyses of Maver-1 and DOHH2 cells. DR and DQ peptidomes from Maver-1 and DOHH2 cells were acquired in DIA mode in triplicates and analysed

using Skyline. The graph displays the number of unique entries in the spectral libraries (light grey) and the number of peptides identified in a triplicate DIA analysis with an mProphet q-value below 0.01 (dark grey). (**C**) Reproducibility of Maver-1 and DOHH2 DIA peptidome analysis. Triplicate DIA DR and DQ peptidome analyses of Maver-1 and DOHH2 cells were analysed using Skyline and filtered to 1% FDR with mProphet. Euler-Venn diagrams display the overlap of peptide identifications between replicates. (**D**) Comparison of sensitivity between DDA and DIA with varying input cell numbers. DR peptidomes were isolated from Maver-1 lysate corresponding to 1, 5, 10, 25 and 100 million Maver-1 cells. Samples were acquired in either DDA or DIA mode in triplicates. DDA data was analyzed with SEQUEST and results filtered for 1% FDR with Percolator. DIA data was analyzed with Skyline using the Maver-1 DR library described in (**B**) and the FDR was estimated with mProphet and set to 1% at peptide precursor level. The graph shows the means and corresponding standard deviations of unique peptides identified in DDA mode (dark grey) and DIA mode (light grey) for the different input amounts.

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Table I

Putative HLA DR-derived peptide sequences identified from plasma of four donors (D1-D4).

Uniprot Accession	Protein Name	Gene Name	Aligned Peptide Sequence	D1	D2	D3	D4	Collado[1]	Ciudad[2]	Maver-1	DOHH2
P01023	Alpha-2-macroglobulin	A2M	SSKFQVDNNNRLL				X		Donor A, B (DRB1*03:01)		
P01833	Polymeric immunoglobulin receptor	PIGR	ASVDSGSSEEQGGSSRA –SVDSGSSEEQGGSSRA	X X							
P02808	Statherin	STATH	DSSEEKFL– DSSEEKFLR	X X							
P04406	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	GKVKVGVNGFGRIGR– GKVKVGVNGFGRIGRL	X	X		X				X
P05186	Alkaline phosphatase, tissue-nonspecific isozyme	ALPL	AHNNYQAQSAVPLRHE			X		t1 (DRB1*01:01)			
P06280	Alpha-galactosidase A	GLA	GPRSYTIAVASLGKG			X			Donor D (DRB1*01:01)	X	X
P07339	Cathepsin D	CTSD	YTVFDRDNNRVGFAEAAR	X	X						X
P07355	Annexin A2	ANXA2	RDALNIETAIKTKG			X			Donor D (DRB1*01:01)	X	
P11279	Lysosome-associated membrane glycoprotein 1	LAMP1	LNTILPDARDPAFK				X		Donor A, B (DRB1*03:01)		
P17936	Insulin-like growth factor-binding protein 3	IGFBP3	HSKIIIIKKGHAKD– – HSKIIIIKKGHAKDSQ				X X	t4 (DRB1*03:01)			
P21333	Filamin-A	FLNA	EETVITVDTKAAGKGK –ETVITVDTKAAGKGK				X X		Donor A (DRB1*03:01, DRB1*11:01)		
P61769	Beta-2-microglobulin	B2M	RTPKIQVYSRHPAENGK –TPKIQVYSRHPAE– – – –TPKIQVYSRHPAEN– – –TPKIQVYSRHPAENGK			X X X X		t5 (DRB1*11:04)	Donor B (DRB1*01:01)	X X X X	
Q02413	Desmoglein-1	DSG1	VATDLDTGRPSTTVR– VATDLDTGRPSTTVRY	X X							
Q9H3G5	Probable serine carboxypeptidase CPVL	CPVL	AGKYVPAIAHLIH– AGKYVPAIAHLIHS – –KYVPAIAHLIH– – –KYVPAIAHLIHS			X X X X		t1 (DRB1*01:01) t2 (DRB1*01:01)	Donor G (DRB1*07:01, DRB1*15:01) Multiple Donors	X	

[1]Peptide sequence identified by Collado et al. [39]

[2]Peptide sequence identified by Ciudad et al. [40]

Table II

Putative HLA DQ-derived peptide sequences identified from plasma of four donors (D1-D4).

Uniprot Accession	Protein Name	Gene Name	Aligned Peptide Sequence	D1	D2	D3	D4	Bergseng[1]
O15127	Secretory carrier-associated membrane protein 2	SCAMP2	TFHRAASSAAQGAFQGN		X			X
			– –HRAASSAAQGAFQGN		X			
O95810	Caveolae-associated protein 2	CAVIN2	TKIVSVERREKIK– – –	X		X		
			TKIVSVERREKIKKSL			X		
			RPPGFSPFR		X		X	
P01127	Platelet-derived growth factor subunit B	PDGFB	HTHDKTALKETLG–		X			
			HTHDKTALKETLGA		X	X		
P01903	HLA class II histocompatibility antigen, DR alpha chain	HLA-DRA	ASFEAQGALANIAVDK–		X			X
			ASFEAQGALANIAVDKA		X	X		X
			– –SFEAQGALANIAVDK–		X			X
			– – –EAQGALANIAVDK–		X	X		X
			– – –EAQGALANIAVDKA			X		X
			TGVSETVFLPREDH	X				X
P04114	Apolipoprotein B-100	APOB	KTTKQSFDLSVKAQYKKNKHRHS		X			
			–TTKQSFDLSVKAQYKKNKHRHS		X			
P04233	HLA class II histocompatibility antigen gamma chain	CD74	KPPKPVSKMRMATPLLMQA			X		X
P04406	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	GKVKVGVNGFGRIGR–	X		X		
			GKVKVGVNGFGRIGRL		X			
P05019-2	Isoform 2 of Insulin-like growth factor I	IGF1	HTDMPKTQKEVHLKNASRGSAGNKN– – –			X		
						X		
				X	X	X	X	
			HTDMPKTQKEVHLKNASRGSAGNKNY– –					
			HTDMPKTQKEVHLKNASRGSAGNKNYRM					
P07339	Cathepsin D	CTSD	YTVFDRDNNRVGFAEAAR	X	X	X		X
P10124	Serglycin	SRGN	SLDRNLPSDSQDLGQHGLEED		X			X
P16109	P-selectin	SELP	HLGTYGVFTNAA– – – – –		X			
			HLGTYGVFTNAAFDPSP		X	X		
P21796	Voltage-dependent anion-selective channel protein 1	VDAC1	AGGHKLGLGLEFQA		X			X
P30443	HLA class I histocompatibility antigen, A-1 alpha chain	HLA-A	EDLRSWTAADMAAQ–	X				X
			EDLRSWTAADMAAQI	X				
P30460	HLA class I histocompatibility antigen, B-8 alpha chain	HLA-B	EDLRSWTAADTAAQ– –	X				X
			EDLRSWTAADTAAQI–	X				X
			EDLRSWTAADTAAQIT	X				X
P45880	Voltage-dependent anion-selective channel protein 2	VDAC2	AGGHKVGLALELEA		X			X
Q05682	Caldesmon	CALD1	STHQAAIVSKIDSRLE	X	X	X	X	
			– –HQAAIVSKIDSRLE	X	X	X	X	

[1] Peptide sequence identified by Bergseng et al. [14]