Sampling From the Proteome to the Human Leukocyte Antigen-DR (HLA-DR) Ligandome Proceeds Via High Specificity*

Geert P. M. Mommen†§¶**, Fabio Marino§¶ ¶¶, Hugo D. Meiring†, Martien C. M. Poelen†, Jacqueline A. M. van Gaans-van den Brink†, Shabaz Mohammed§¶¶‡‡, Albert J. R. Heck§¶¶, and Cécile A. C. M. van Els

Comprehensive analysis of the complex nature of the Human Leukocyte Antigen (HLA) class II ligandome is of utmost importance to understand the basis for CD4⁺ T cell mediated immunity and tolerance. Here, we implemented important improvements in the analysis of the repertoire of HLA-DR-presented peptides, using hybrid mass spectrometry-based peptide fragmentation techniques on a ligandome sample isolated from matured human monocyte-derived dendritic cells (DC). The reported data set constitutes nearly 14 thousand unique high-confident peptides, i.e. the largest single inventory of human DC derived HLA-DR ligands to date. From a technical viewpoint the most prominent finding is that no single peptide fragmentation technique could elucidate the majority of HLA-DR ligands, because of the wide range of physical chemical properties displayed by the HLA-DR ligandome. Our in-depth profiling allowed us to reveal a strikingly poor correlation between the source proteins identified in the HLA class II ligandome and the DC cellular proteome. Important selective sieving from the sampled proteome to the ligandome was evidenced by specificity in the sequences of the core regions both at their N- and C- termini, hence not only reflecting binding motifs but also dominant protease activity associated to the endolysosomal compartments. Moreover, we demonstrate that the HLA-DR ligandome reflects a surface representation of cell-compartments specific for biological events linked to the maturation of monocytes into antigen presenting cells. Our results present new perspectives into the complex nature of the HLA class II system and will aid future immunological studies in characterizing the full breadth of potential CD4⁺ T cell epitopes relevant in health and disease. Molecular & Cellular Proteomics 15: 10.1074/mcp.M115.055780, 1412–1423, 2016.

Human Leukocyte Antigen (HLA) class II molecules on professional antigen presenting cells such as dendritic cells (DC) expose peptide fragments derived from exogenous and endogenous proteins to be screened by CD4⁺ T cells (1, 2). The activation and recruitment of CD4⁺ T cells recognizing disease-related peptide antigens is critical for the development of efficient antipathogen or antitumor immunity. Furthermore, the presentation of self-peptides and their interaction with CD4⁺ T cells is essential to maintain immunological tolerance and homeostasis (3). Knowledge of the nature of HLA class II-presented peptides on DC is of great importance to understand the rules of antigen processing and peptide binding motifs (4), whereas the identity of disease-related antigens may provide new knowledge on immunogenicity and leads for the development of vaccines and immunotherapy (5, 6).

Mass spectrometry (MS) has proven effective for the analysis HLA class II-presented peptides (4, 7, 8). MS-based ligandome studies have demonstrated that HLA class II molecules predominantly present peptides derived from exoge-
nous proteins that entered the cells by endocytosis and endogenous proteins that are associated with the endo-lysosomal compartments (4). Yet proteins residing in the cytosol, nucleus or mitochondria can also be presented by HLA class II molecules, primarily through autophagy (9–11). Multiple studies have mapped the HLA class II ligandome of antigen presenting cells in the context of infectious pathogens (12), autoimmune diseases (13–17) or cancer (14, 18, 19), or those that are essential for self-tolerance in the human thymus (3, 20). Notwithstanding these efforts, and certainly not in line with the extensive knowledge on the HLA class I ligandome (21), the nature of the HLA class II-presented peptide repertoire and particular its relationship to the cellular source proteome remains poorly understood.

To advance our knowledge on the HLA-DR ligandome on activated DC without having to deal with limitations in cell yield from peripheral human blood (12, 21, 22) or tissue isolates (3), we explored the use of MUTZ-3 cells. This cell line has been used as a model of human monocyte-derived DCs. MUTZ-3 cells can be matured to act as antigen presenting cells and express then high levels of HLA class II molecules, and can be propagated in vitro to large cell densities (23–25). We also evaluated the performance of complementary and hybrid MS fragmentation techniques electron-transfer dissociation (ETD), electron-transfer/higher-energy collision dissociation (EThCD) (26), and higher-energy collision dissociation (HCD) to sequence and identify the HLA class II ligandome. Together this workflow allowed for the identification of an unprecedented large set of about 14 thousand unique peptide sequences presented by DC derived HLA-DR molecules, providing an in-depth view of the complexity of the HLA class II ligandome, revealing underlying features of antigen processing and surface-presentation to CD4⁺ T cells.

**EXPERIMENTAL PROCEDURES**

**Ethics**—Institutional principles of RIVM relating to the use of material and data obtained from human subjects, including prototype cell lines obtained via cell bank catalogues, are in agreement with the guidelines expressed for Good Clinical Practice expressed in the Declaration of Helsinki.

**Cell Culturing, Lysis of Cells and Isolation of HLA Class II-associated Peptides**—The deposited MUTZ-3 cell line, a human HLA-DR10, -DR11, -DR52 (HLA-DRB1*10, HLA-DRB1*11, HLA-DRB3*01) positive acute myelo-monocytic leukemia serving as a dendritic cell model (kindly provided by Dr. R. Scheeper, VU University Medical Center, Amsterdam), was grown under maintenance conditions in roller bottles in α-Minimum Essential Medium (Gibco, Thermo Fisher Scientific, Bremen, Germany), supplemented 20% heat-inactivated FBS (HyClone, Logan, USA), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), 2 mM l-glutamine (Gibco), and 25 μg/ml GM-CSF (Peptotech, London, UK)¹. MUTZ-3 cells were induced into an immature DC state by a 5-day exposure to 1000 U/ml GM-CSF (100 ng/ml), 1000 U/ml IL-4 (20 ng/ml) and 2.5 ng/ml TNF-α. Immature MUTZ-3 DC were matured by increasing the concentration of TNF-α to 75 ng/ml for 20 h. During this maturation phase BCG antigens were present as an antigenic pulse (kindly provided by Camille Locht, Institut Pasteur de Lille, France). The mature state was verified based on the expression of DC-associated maturation markers CD40, CD80, CD83, and CD86 by flow cytometry (data not shown). The large bulk of 1.2 × 10⁹ cells stimulated MUTZ-3 was washed in ice cold PBS and snap frozen before lysing and solubilizing of cell membrane proteins with Nonidet P40 containing IP lysisbuffer (Thermo Fisher Scientific). After removal of the nonsolubilized fraction using ultracentrifugation, HLA class II molecules were immunoprecipitated from the MUTZ-3 cell lysate using the HLA-DR-specific monoclonal antibody L243. An aliquot of the MUTZ-3 cell lysate after HLA-DR pull down was used for proteomics. HLA class II molecules and associated peptides were eluted with 10% acetic acid and peptides were collected by passage over a 10-kDa mw cutoff membrane and concentrated using vacuum centrifugation.

**MUTZ-3 Cell Lysate Digestion**—The MUTZ-3 cell lysate was diluted in 2 ml urea, 50 mM ammonium bicarbonate containing one tablet of EDTA-free protease inhibitor mixture (Sigma-Aldrich, Zwijndrecht, The Netherlands) and one tablet of PhosSTOP phosphatase inhibitor mixture (Roche, Almere, The Netherlands). Cysteine residues were reduced and alkylated using 200 mM dithiotreitol (Sigma-Aldrich) and 200 mM iodoacetamide (Sigma-Aldrich). The proteins were digested with Lys-C (Roche) at an enzyme/protein ratio of 1:75 for 4 h at 37 °C. Two times diluted samples were digested with trypsin (Roche) overnight at 37 °C at an enzyme/protein ratio of 1:100. Peptide mixtures were desalted using a 1-cc Sep Pac C18 columns (Waters, Etten-Leur, The Netherlands) according manufacturer’s protocol.

**Fractionation of HLA-DR Ligands and Tryptic Peptides**—HLA-DR eluted peptides were fractionated by strong cation exchange (SCX) chromatography (27). The system consists of a Hypercarb™ trapping column (200 μm i.d., 5 mm, 7 μm particle size, Thermo Fisher Scientific) and SCX analytical column (200 μm i.d., 12 cm, polysulfoethyl aspartamide, 5 μm, PolyLC, Columbia, USA). The peptides were separated by a linear salt gradient ramping to 500 mM KCl in 0.1 M HOAc and 35% acetonitrile at a column flow rate of 2 μl/min. A total number of 26 fractions (2 min per fraction) were collected, dried down using a vacuum centrifuge, and reconstituted. Based on the LC-MS/MS signal intensities of pre-analyzed sample aliquots the 10 most informative fractions were selected for analysis.

Tryptic peptides from the MUTZ-3 digest were fractionated by SCX using a ZorbaxBioSCX-Series II column (0.8 mm i.d., 50 mm, 3.5 μm particle size, Agilent Technologies, Waldbronn, Germany). A multistep gradient up to 500 mM NaCl in 0.05% formic acid 20% acetonitrile was used to separate the tryptic peptides (28). Fractions were pooled based on their UV signal intensity to a total of 10 fractions.

**Reversed Phase Liquid Chromatography and Mass Spectrometry**—For the HLA ligands, each individual SCX fraction was analyzed in triplicate by nanoscale LC-MS using a Thermo Scientific EASY-nLC 1000 (Thermo Fisher Scientific) and ETD enabled LTQ Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) with either EThCD, HCD or ETD fragmentation. The system comprises an in-house packed 200 μm i.d., 100 μm o.d. trapping column (Reprosil C18, 3 μm, Thermo Fisher Scientific) and a 50 cm EASY-nLC column (200 μm i.d., 5 μm, Dr Maisch, Ammerbuch, Germany) and one tablet of PhosSTOP phosphatase inhibitor mixture (Roche, Almere, The Netherlands) and one tablet of PhosSTOP phosphatase inhibitor mixture (Roche, Almere, The Netherlands). Two times diluted samples were digested with trypsin (Roche) overnight at 37 °C at an enzyme/protein ratio of 1:100. Peptide mixtures were desalted using a 1-cc Sep Pac C18 columns (Waters, Etten-Leur, The Netherlands) according manufacture’s protocol.

² For EThcD, modified instrument firmware was used to allow all-ion scanning. The total gain control (AGC) target in MS/MS was set to 3 × 10⁴. For MS scans was set to 250 ms and for MS/MS scans to 1500 ms. For EThcD, modified instrument firmware was used to allow all-ion scanning.
Hybrid MS-based Identification of the HLA Class II Ligandome

HCD fragmentation after an initial ETD. The HCD normalized collision energy was set to 32%. The ETD reaction time was set to 50 ms and supplemental activation and charge dependent activation time was enabled. Precursor ions with unknown and +1 charge states were excluded from MS/MS analysis. Dynamic exclusion was enabled (exclusion size list 500) with a repeat count of 1 and an exclusion duration of 60 s.

The SCX fractions of the tryptic digested MUTZ-3 cells were analyzed by LC-MS/MS using an Agilent 1290 Infinity System (Agilent Technologies) modified for nanoflow LC (passive split) connected to a TripleTOF analyzer (Sciex, Nieuwekerk aan den IJssel, The Netherlands). Peptides were eluted using a similar trapping and analytical column system and LC gradient conditions as described above. A voltage of 2.7 kV was applied to the needle. The survey scan was from 375 to 1250 m/z and the high resolution mode was utilized, reaching a resolution of up to 40,000. Tandem mass spectra were acquired in high sensitivity mode with a resolution of 20,000. The 20 most intense precursors were selected for subsequent fragmentation using an information dependent acquisition, with a minimum acquisition time of 50 ms.

Data Analysis—The raw files collected from the TripleTOF were first recalibrated based on five background ions with m/z values of 391.2847, 445.12003, 519.13882, 593.15761, 667.17640. The calibrated raw files were converted to mgf by the AB Sciex MS Data Converter (version 1.3 beta) program before analysis with Proteome Discoverer 1.4. RAW files acquired with the Orbitrap Elite were directly analyzed with Proteome Discoverer 1.4 software package (Thermo Fisher Scientific) using default settings unless otherwise stated. For the EThcD and ETD spectra the nonfragment filter was added with the following settings: The precursor peak was removed stated. For the EThcD and ETD spectra the nonfragment filter was (Thermo Fisher Scientific) using default settings unless otherwise

Cluster-1.0 algorithm (33) was for simultaneous alignment and clustering of complete data set of HLA-DR-associated peptides. Gibbs clustering was performed using default settings (33), with preference of hydrophobic amino acids at position P1, the number of clusters set to 1–4, λ set to 0.8. Sequence logo’s were created using IceLogo (34), with p values set to 0.005. Following accepted procedures the protein/peptide abundances were estimated based on their spectral counts normalized to the protein length (33). The mass spectrometry data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (36) with the data set identifier PXD002951.

RESULTS

Identification of Naturally Presented HLA-DR-peptides—HLA-DR-peptides were isolated from human matured MUTZ-3 DCs and prefractionated by strong cation exchange chromatography prior to analysis by reversed phase LC-MS/MS, employing the complementary peptide fragmentation methods HCD, ETD, and EThcD (Fig. 1A). Table I summarizes the global outcome of the HLA-DR peptide identification results (supplemental Data). Although the highest number of MS/MS events were acquired by HCD, it showed a substantial lower identification rate than ETD and EThcD. In our work by using ETD the largest number of unique peptides could be identified (9431), followed by EThcD (8254), and finally HCD (6725). The combination of the three techniques was essential to expand the ligandome identification results, because only 53% of all peptides were identified by two or more fragmentation techniques (Fig. 1B). The combined results yielded 13,918 unique HLA-DR-peptides originating from just under two thousand (1980) source proteins (Table I).

To check whether peptide identification depended on the peptide’s physicochemical properties, we next evaluated the performance of HCD, ETD, and EThcD in terms of peptide length and charge state. Supplemental Fig. S1 shows that there were moderate differences in the physicochemical properties of the peptides identified by the applied peptide fragmentation techniques. EThcD displayed a preference for smaller (6–11 amino acids in length) and doubly charged peptides, whereas ETD displayed a bias for longer peptides with higher charge state.

The inventory of 13,918 HLA-DR peptides included peptides that contained oxidized methionine, deamidated asparagine, and glutamine, most likely modifications that are associated with sample preparation (613 modified peptides in total). We identified an additional fraction of 916 unique peptides bearing a variety of post translational modifications,
including cysteinylation, N-terminal and lysine acetylation, serine and threonine phosphorylation, and (di)methylation on arginine and lysine residues.

When compared with some of the most extensive previous HLA class II studies in literature, our data set represents a substantial improvement of the number of HLA-DR peptides identified (supplemental Table S1). Despite the limited overlap between peptides identified, likely because of different HLA-DR backgrounds, our study covered a large fraction (~50%) of the previous identified source proteins and extensively increased the list of protein antigens presented by HLA class II molecules.

**Figure 1.** HLA-DR ligandome analysis and characteristics. A, Workflow for the analysis of the HLA-DR-presented ligandome and proteome of MUTZ-3 DC. For the ligandome, following affinity purification of HLA-DR-peptide complexes, peptides were released by acid elution, fractionated by strong cation exchange (SCX) and analyzed by LC-MS/MS using ETD, EThCD, and HCD fragmentation. Global proteome analysis was performed following trypsin digestion of lysed MUTZ-3 cells, using SCX fractionation prior to LC-MS/MS analysis. B, Venn diagram displaying the overlap and unique contribution to the identified HLA-DR ligandome by EThCD, ETD and HCD. C, Peptide length distribution in the combined HLA-DR ligandome (n = 13,918). D, Illustrative nested set of HLA-DR-associated peptides derived from Elongation factor 1-α 1, consisting of 10 peptide length variants with a consensus core binding motif but varying extended N- and C termini.
Hybrid MS-based Identification of the HLA Class II Ligandome

Table I
Comprehensive HLA-DR ligandome analysis of mature MUTZ-3 DC by mass spectrometry using the complementary peptide fragmentation techniques ETD, HCD, and EThcD

| Data set                                      | ETD    | HCD    | EThcD | Total  |
|----------------------------------------------|--------|--------|-------|--------|
| Total # of MS/MS spectra                     | 54,059 | 61,129 | 46,351| 161,539|
| peptide-to-spectrum-matches, PSMs (<1% FDR)  | 14,100 | 10,694 | 12,755| 37,549 |
| identification (%)                           | 26     | 17     | 28    | 23     |
| unique HLA-DR peptides                       | 9431   | 6725   | 8254  | 13,918 |
| unique source proteins                       | 1516   | 1314   | 1280  | 1980   |

Characteristics of the HLA-DR ligandome

| ‘nested sets’                                 | 1472   | 1066   | 1237  | 2060   |
| peptides presented in nested sets            | 7624   | 5382   | 6998  | 11,278 |
| median (and range) of peptide copy numbers per cell | 2 (<1–596) | 1 (<1–382) | 3 (<1–962) | 2 (<1–962) |
| median (and range) of unique HLA-DR peptides per source protein | 2 (1–612) | 2 (1–445) | 2 (1–598) | 2 (1–800) |
| median (and range) of nested sets per source protein | 1 (0–38) | 1 (0–32) | 1 (0–36) | 1 (0–44) |
| median (and range) of cumulative peptide copy number per source protein per cell | 4 (<1–4085) | 2 (<1–1958) | 6 (<1–6610) | 3 (<1–7450) |

Peptides (84%) corresponded to so-called nested sets, having a common binding core extended by flanking N- and/or C-terminal residues (Table I, Fig. 1D). In total, 2060 nested sets were observed, an average of ~1 nested set per protein (Table I). The majority of nested sets were represented by two or three peptides but some consisted of more than 60 variants.

The NetMHCIIpan-3.0 algorithm (32) was used to predict the 9 amino acids core sequence of the peptides that facilitates binding to the expressed HLA-DR10, HLA-DR11 and HLA-DR52 molecules. A total of 10,393 binding cores were predicted to have a moderate to strong affinity to the expressed HLA-DR molecules (< 1000 nM). These predicted binding cores were derived from only 5795 unique peptides, indicating that for many peptide multiple core sequences were predicted to bind to different HLA-DR molecules and no single HLA-DR presenting molecule could be assigned. From the 10,393 predicted binding cores, only 1877 were predicted to bind to a single HLA-DR molecule, whereas the remaining ones displayed unambiguity and/or redundancy having a moderate to strong predicted affinity to multiple HLA-DR molecules. These results likely indicate that the NetMHCIIpan3.0 software lacks specificity to discriminate in binding between the three investigated different HLA-DR molecules. Therefore, we decided to also make use of the Gibbs alignment and clustering algorithm (Gibbs-Cluster-1.0) (33) to extract the binding motifs directly from the complete set of HLA-DR ligands. We clustered the complete set of peptides into four groups, one for each investigated HLA-DR molecule and one nonspecific cluster. The Gibbs-Cluster-1.0 could assign to the different HLA-DR clusters a total of 8569 unique peptides, without overlap between the generated peptide groups.

Fig. 2 shows three sequence logo’s generated by the Gibbs-Cluster-1.0 analysis (Fig. 2D, 2E, 2F), two of which (Fig. 2D, 2F) agree reasonably well with the sequence logo’s of the NetMHCIIpan3.0 predictions for HLA-DR10 and HLA-DR52 (respectively Fig. 2A, 2C). The sequence logo’s of the predicted binding motifs exhibited marked differences between HLA-DR10, HLA-DR11, and HLA-DR52 (Fig. 2) at the P1, P4, P6, and P9 anchor residues. All three expressed HLA-DR molecules had a clear selectivity for both aliphatic and aromatic hydrophobic residues such as Phe, Tyr, Leu, and Ile at one or more of the anchor positions. Although aspartic acid (Asp) was found exclusively enriched at anchor position P4 for HLA-DR52, the Gibbs cluster analysis indicated that the negatively charged glutamic acid (Glu) was preferred on P3, P4, and P5 as well. For all three HLA-DR molecules we found that the positively charged residues Lys and Arg were moderately enriched at the positions P2, P3, P5, and P8. This observation was confirmed by the Gibbs cluster analysis.

Cleavage Specificity of Proteases Involved in HLA-DR Ligand Processing—To determine which endo-lysosomal proteases may be involved in the generation of the HLA-DR ligands, we analyzed the distribution of amino acids at the cleavage sites at both the N terminus and the C terminus of the ligands. Fig. 3 shows the over- and under-represented amino acids flanking the cleavage site both at the N terminus (left panel) and the C terminus (right panel). For both termini, cleavage preferentially occurs between hydrophobic and acidic residues. The high preference for Leu, Phe and Asp at P1 of the cleavage site hints to a dominant role of the proteases Cathepsin D or Cathepsin E (37, 38). In our subsequent global proteome analysis of MUTZ-3 cells (Fig 1A) a prominent expression of Cathepsin D compared with other proteases involved in the endo-lysosomal degradation, could be established (supplemental Table S2). The proteomic analysis also revealed the presence of several other proteases possibly involved in the ligand processing, such as Cathepsin B, H, S, Z, albeit generally detected at lower abundance than Cathepsin D. Therefore, Cathepsin D is likely the most prominent...
protease involved in the processing of MHC II molecules in MUTZ-3 cells.

The specificity profiles also showed amino acid residues that were under-represented at the cleavage site (Fig. 3, lower parts of logo’s). The small amino acids proline and serine were identified as poor substrates for the enzymes involved in antigen processing. Although proline residues were generally under-represented surrounding the cleavage sites, we found enrichment of proline residues close to the N-terminal end of HLA-DR ligands. Enrichment of proline residues located penultimate to the N-terminal cleavage has been linked to the blocking of N-terminal trimming in HLA class II peptides (39). This observation, linked to the identification of Aminopeptidase-N in both the global proteome and the HLA-DR ligandome, hints at a possible role for this enzyme in HLA class II processing in MUTZ-3 (supplemental Table S2, Fig. 4D).

High dominance in the HLA-DR binding groove—The median number of HLA-DR-peptides identified per source protein was 2, but varied widely ranging from a single peptide up to 800 unique peptides (Table I). The latter high number was found to be largely because of very extensive nested set formation. To obtain a (semi) quantitative impression of the HLA-DR sampling process, we calculated the peptide copy number per cell using known amounts of two synthetic pep-
Tides as reference which were spiked in each sample fraction prior to LC-MS analysis. These calculated copy numbers may however not accurately reflect the actual presentation levels because we do not account for losses during sample preparation and variation in MS detection (e.g., ionization efficiency).

We estimated that HLA-DR peptides were presented on MUTZ-3 cells with a median of 2 copies/cell with a range between 1 and 962 copies/cell, indicating a dynamic range of four orders of magnitude in the display of potential individual CD4 T cell epitopes (Table I). The surface presentation levels of the source proteins were determined by summing the copy per cell numbers of their representative peptides. The cumulative peptide copy number per protein per cell ranged from 1–7450 (5 orders of magnitude) (Table I), which follows directly from the high diversity in number of peptides presented per protein (i.e., nested sets). Based on these copy numbers, we estimated that the 100 most frequently sampled proteins already occupy ~80% of the HLA-DR molecules, whereas the remaining 1881 proteins account for only ~20% of the quantitative HLA-DR ligandome.

Selective Sampling of the Cellular Proteome by HLA-DR—

The most frequently HLA-DR-sampled proteins included actins, myosins, aminopeptidase N and the HLA class II β chain itself (Fig. 4D, 4E). These proteins are known to be involved in cytoskeletal organization and antigen processing and presentation during maturation of monocytes into dendritic cells after stimulation (40, 41). We used PANTHER (42) for Gene Ontology (GO) classification of the HLA-DR ligandome, whereby the concomitantly analyzed MUTZ-3 cellular proteome was used as a reference set (Fig. 4B). Analysis of the GO terms of the source proteins of the HLA-DR ligandome against the background of the global MUTZ-3 proteome (rather than against the full human proteome) revealed an MHC class II pathway-specific signature in GO annotation for stimulated DCs. Compared with the cellular proteome, the HLA-DR ligandome was enriched in proteins involved in cell adhesion, immune system processes and system development (Supplemental Table 3). CELLO2GO (31) was used to predict protein cellular localization of the source proteins detected in the HLA-DR ligandome and the global cellular proteome (Fig. 4B). A major fraction of the ligandome source proteins originated from the nuclear compartment (36%), followed by the cytoplasm (25%) and the plasma membrane (17%). Compared with the MUTZ-3 global proteome, proteins contributing to the ligandome were relatively enriched in membrane associated proteins and proteins from the extracellular matrix, whereas proteins localized in the nucleus and cytoplasm were underrepresented.

Finally, we investigated the relation between the HLA-DR ligandome and the global MUTZ-3 proteome with respect to the identified proteins and their associated number of peptides. Global proteome analysis provided abundance estimates for about 3300 proteins (Fig. 4A). Only 1195 proteins (35%) were also identified as source proteins in the HLA-DR ligandome (Fig. 4C). Moreover, we observed that certain source proteins that were represented by a high number of
Fig. 4. Source proteome of the HLA-DR ligandome. A, The cumulative number of unique peptides as function of the number of proteins identified by global proteome (blue) and the HLA-DR ligandome (red) analysis. Proteins are ranked from highest to the lowest number of representative peptides. B, The CELLO2GO algorithm was used to predict the subcellular localization of source proteins obtained by HLA-DR ligandome (blue bars) and global cellular proteome (red bars) analysis. C, Venn diagram showing the overlap between source-proteins identified in the HLA-DR ligandome and the full proteome. D, Source proteins most frequently sampled by HLA-DR with the number of observed unique HLA-DR peptides and number of nested sets. E, Dynamic range plot of the global cellular proteome of MUTZ-3 (blue dots). The assigned proteins (red dots) were the 19 out of 25 most abundantly expressed proteins in the HLA-DR ligandome (6 proteins HLA-DR-sampled proteins were not identified by global proteome analysis).
Hybrid MS-based Identification of the HLA Class II Ligandome

HLA-DR peptides were absent in the global cellular analysis of MUTZ-3 cells, and vice versa, which could point to a rather weak correlation between expression levels of the HLA-DR ligandome and global proteome. To illustrate this, we determined the correlation between the proteins most frequently sampled by HLA-DR and their cellular expression levels (global proteome analysis) using a recently described peptide count approach (41). Fig. 4E shows that the most abundantly observed proteins in the HLA-DR ligandome are distributed over the full dynamic range plot of the source proteome.

DISCUSSION

Natural processing and Human Leukocyte Antigen (HLA) class II presentation of extracellular and intracellular proteins are key yet still unpredictable functions of activated dendritic cells (DC) that steer and regulate adaptive immune responses. Advances in understanding the outcome of these processes require comprehensive analysis of endogenously processed and surface presented peptide repertoires, demanding cutting edge technology (43). To expand the mass spectrometry-based identification of HLA class II presented peptides we combined the (hybrid) peptide fragmentation techniques, HCD, ETD, and EThcD, on a large scale preparation of HLA-DR peptides derived from an in vitro matured human prototype monocyte-derived DC cell line, MUTZ-3. Cumulatively, the exceptionally high number of 13,918 unique identified HLA-DR peptides, a significant improvement in the number of ligands compared with previous endeavors (18, 22, 44, 45), gave unprecedented insight into various underlying features of selectivity in HLA-DR ligandome.

MS-based identification of HLA class II-associated peptides by nonstandard ETD or EThcD based sequencing was until now little explored. Our data revealed an improved rate of peptide identification for techniques involving electron transfer dissociation over classical collision induced dissociation for HLA-DR peptides, confirming previous reports on the analysis of endogenous complexes (46, 47). HLA-DR-presented peptides were highly variable in nature, especially in their wide distribution in peptide length and charge state. ETD had a slight preferential bias for longer and/or higher charged HLA-DR-peptides (48, 49). Earlier, using identical nonstandard MS technology in an HLA class I peptide inventory (50) we identified 12,199 unique HLA class I peptides originating from 5,603 proteins in a human B lymphoblastoid cell line (i.e. on average ~2 peptides per protein). In our present comprehensive survey addressing the HLA class II pathway we identified 13,918 HLA-DR ligands derived from only 1980 proteins (i.e. on average ~7 peptides per protein). This much more sparse source proteome, together with the observation of many nested sets of length variants of the same epitope region, suggests that the overall HLA-DR epitope landscape available for surveillance by circulating CD4+ T cell populations is less diverse than the HLA class I ligandome inspected by CD8+ T cells.

The source proteins sampled by HLA-DR molecules reflected only a minor part (~35% overlap) of the global proteome identified in the MUTZ-3 cell lysate. Vice versa, some most frequently sampled proteins in the HLA-DR ligandome were absent from the detectable MUTZ-3 proteome, which rather suggests a poor correlation between expression levels in the global proteome and protein representation in the HLA-DR ligandome. For the intrinsically different HLA class I antigen presentation route, multiple reports have established that it is difficult to predict ligand copy numbers from overall protein or RNA levels (21, 51–53), perhaps with the exception of proteins with high turn-over (35). Therefore, most studies including ours substantiate the selective sampling of proteome cargo for both HLA class I and class II molecules on their way to the cell surface. Indeed, GO analysis revealed that the surface-presented HLA-DR ligandome of MUTZ-3 DCs was biased for proteins involved in cell mobility, cytoskeletal re-organization, HLA class II processing and presentation, functions strongly linked to the maturation of monocytes into antigen presenting cells (40, 41). The MUTZ-3 DCs in our study were matured via TNFα, an endogenous “danger” signal that can induce the transition from an antigen capturing DC to an antigen presenting DC (54). Relative enrichment was also found for proteins associated with the membrane and extracellular matrix, the main contributors to the lysosomal degradation and HLA class II presentation pathway (55). Direct or indirect evidence for the dominance of membrane and extracellular proteins as source proteins for MHC class II ligandomes has been implied before (20, 56–58), in line with the mechanisms of endocytosis, lysosomal trafficking and processing, preceding MHC class II loading with peptides. Even while being underrepresented relative to the global MUTZ-3 proteome, proteins associated with the nuclear compartment or cytoplasm made up to 50% of the source proteins of the HLA-DR ligandome. We interpret this to be the outcome of autophagy, a stress response process shuttling large fractions of intracellular proteins into the lysosomal compartment promoting their presentation by HLA class II (59). Earlier autophagy has been implied to be essential for HLA-DR loading with intracellular material relevant for CD4+ T cells to monitor cellular virus infection (60, 61), transformation (62–64) or stress (65), or to shape their self-tolerance (10, 66, 67).

The observation that the most abundantly sampled source proteins occupied with their peptide sequences already ~80% of the HLA-DR molecules, further indicates that in the antigen selection process certain proteins have strong advantage of being presented over others. The dynamic range of HLA-DR ligandome ranged from less than a single peptide copy per protein to extremes where cumulatively almost 7500 peptide copies per protein were detected per cell. Strong peptide hierarchies have been seen in the HLA class I ligandome (68) and human and murine MHC class II ligandomes of
The ligands associated with the HLA-DR complex with the T-cell receptor. Novellino play an important role in the interaction of the HLA-DR-peptide motif. The positively charged amino acids flanking the anchor residues could be associated with peptides has been reported previously (45). Positively charged amino acids in the P8 positions of HLA-DR10-restricted peptide YFAAELPPRN lost recognition when arginine was substituted by glycine. Likely, ETD and ETHcD have contributed to the unambiguous assignment of these positively charged amino acids as these fragmentation techniques are specifically beneficial for peptides harboring internal basic residues (49, 74).

In conclusion, complementary peptide fragmentation techniques allow to be used in profiling of human DC derived HLA class II-associated peptides. Our results, based on a single MUTZ-3 HLA-DR ligandome, paved the way for further exploration of HLA class II peptide repertoires under different maturation and antigenic conditions. Exposure of peptides at a wide dynamic range of abundances yet with a relatively limited diversity through strong selective mechanisms seem hallmark of this pathway. Whether these here-observed characteristics of antigen processing and presentation are widely applicable to the HLA class II pathway in human DCs needs further investigation of various DC models and primary cell cultures. The challenge in future studies will be to quantitatively and qualitatively characterize the full breadth of potential CD4+ T cell epitopes relevant in health and disease, especially if hidden as minor specificities among the larger self ligandome.

This work was supported in part by the project Proteins at Work (project 184.032.201), a program of the Netherlands Proteomics Centre financed by the Netherlands Organization for Scientific Research (NWO) as part of the National Roadmap Large-scale Research Facilities of the Netherlands, and by the strategic research projects Immunoproteomics and Correlates of Protection, financed by the Dutch Ministry of Health. This work was also supported by the Institute for Chemical Immunology, an NWO Gravitation project funded by the Ministry of Education, Culture and Science of the Netherlands. The NWO is also kindly acknowledged for financial support with the VIDI grant for Shabaz Mohammed (700.10.429).

This article contains supplemental material. To whom correspondence should be addressed: Antonie van Leeuwenhoeklaan 9, 3721 MA Bilthoven, The Netherlands. Tel.: +31-30-2743665; E-mail: cecile.van.els@rivm.nl.

These authors contributed equally to this work.

Both authors share senior authorship.

REFERENCES

1. Neefjes, J., Jongsm, M. L., Paul, P., and Bakke, O. (2011) Towards a systems understanding of MHC class I and MHC class II antigen presentation. Nat. Rev. Immunol. 11, 833–836
2. Roche, P. A., and Furuta, K. (2015) The ins and outs of MHC class II-mediated antigen processing and presentation. Nat. Rev. Immunol. 15, 203–216
3. Adamopoulos, E., Tenzer, S., Hillen, N., Klug, P., Rota, I. a, Tietz, S., Gebhardt, M., Stevanovic, S., Schild, H., Tolosa, E., Melms, A., and Stoeckle, C. (2013) Exploring the MHC-peptide matrix of central tolerance in the human thymus. Nat. Commun. 4, 2039
4. Suri, A., Lovitch, S. B., and Unanue, E. R. (2006) The wide diversity and complexity of peptides bound to class II MHC molecules. Curr. Opin. Immunol. 18, 70–77
5. Ovsyannikova, I. G., Johnson, K. L., Bergen, H. R., and Poland, G. A. (2007) Mass spectrometry and peptide-based vaccine development. Clin. Pharmacol. Ther. 82, 644–652
6. Thibodeau, J., Bourgeois-Daiglneaut, M. C., and Lapointe, R. (2012) Targeting the MHC Class II antigen presentation pathway in cancer immunotherapy. Oncoimmunology 1, 908–916
7. Purcell, A. W. (2004) Isolation and characterization of naturally processed MHC-bound peptides from the surface of antigen-presenting cells. Methods Mol. Biol. 251, 291–306
8. Lippolis, J. D., White, F. M., Marto, J. A., Luckey, C. J., Bulllock, T. N., Shabanowitz, J., Hunt, D. F., and Engelhard, V. H. (2002) Analysis of MHC class II antigen processing by quantitation of peptides that constitute nested sets. J. Immunol. 169, 5089–5097
9. Dengjel, J., Schoor, O., Fischer, R., Reich, M., Kraus, M., Müller, M., Kreyberg, K., Altenberend, F., Brandenburg, J., Kalbacher, H., Brock, R., Diessen, C., Rammensee, H.-G., and Stevanovic, S. (2005) Autophagy promotes MHC class II presentation of peptides from intracellular source proteins. Proc. Natl. Acad. Sci. U.S.A. 102, 7922–7927
10. Münz, C. (2012) Antigen processing for MHC class II presentation via autophagy. Front. Immunol. 3, 1–6
11. Deretic, V., Saltoh, T., and Akira, S. (2013) Autophagy in infection, inflammation and immunity. Nat. Rev. Immunol. 13, 722–737

Molecular & Cellular Proteomics 15.4
Hybrid MS-based Identification of the HLA Class II Ligandome

12. Stenger, R. M., Meiring, H. D., Kuipers, B., Poelen, M., van Gaans-van den Brink, J. A., Boog, C. J., de Jong, A. P., and van Els, C. A. (2014) Bordetella pertussis proteins dominating the major histocompatibility complex class II-presented epitope repertoire in human monocytic-de- derived dendritic cells. Clin. Vaccine Immunol. 21, 641–650

13. Bergseng, E., Dørum, S., Arntzen, M. Ø., Nielsen, M., Nygård, S., Buus, S., de Souza, G. a., and Solidi, L. M. (2014) Different binding motifs of the cell wall disease-associated HLA molecules DQ2.2, DQ2.2, and DQ7 revealed by relative quantitative proteomics of endogenous peptide repertoires. Immunogenetics 67, 73–84

14. Deponte, F. R., Qian, J., Zaring, A. L., McMiller, T. L., Salay, T. M., Norris, A., English, A. M., Shabanowitz, J., Engelhard, V. H., Hunt, D. F., and Topalian, S. L. (2009) Identification of tumor-associated, MHC class II-restricted phosphopeptides as targets for immunotherapy. Proc. Natl. Acad. Sci. U.S.A. 106, 12073–12078

15. Benham, H., Nel, H. J., Law, S. C., Mehdi, A. M., Street, S., Ramnauth, N., Pahau, H., Lee, B. T., Ng, J., G Brunck, M. E., Hyde, C., Trouw, L. A., Dudek, N. L., Purcell, A. W., O’Sullivan, B. J., Connolly, J. E., Paul, K. S., Lé Cao, K. A., and Thomas, R. (2015) Citrullinated peptide dendritic cell immunotherapy in HLA risk genotype-positive rheumatoid arthritis patients. Sci. Transl. Med. 7, 290ra87

16. Fissolo, N., Haag, S., de Graaf, K. L., Drews, O., Stevanovic, S., Rammensee, H. G., and Weissert, R. (2009) Naturally presented peptides on major histocompatibility complex I and II molecules eluted from central nervous system of multiple sclerosis patients. Mol. Cell. Proteomics 8, 2090–2101

17. Hill, J. a, Southwood, S., Sette, A., Jevnikar, A. M., Bell, D. a, and Cairns, E. (2003) Cutting edge: the conversion of arginine to citrulline allows for a hLA-restricted peptide interaction with rheumatoid arthritis-associated HLA-DRB1*0401 MHC class II molecule. J. Immunol. 171, 538–541

18. Chomoguz, O., Gapeev, a., O’Neill, M. C., and Ostrand-Rosenberg, S. (2012) Major Histocompatibility Complex Class II - Invariant Chain Chaperone in Breast Cancer Cells Present Unique Peptides That Activate Tumor-specific T Cells From Breast Cancer Patients. Mol. Cell. Proteomics, 1457–1467

19. Dengjel, J., Decker, P., Schoor, O., Altenbernd, F., Weischneg, T., Rammensee, H. G., and Stevanovic, S. (2004) Identification of a naturally processed cyclin D1 T-helper epitope by a novel combination of HLA class II targetting and differential mass spectrometry. Eur. J. Immunol. 34, 3644–3651

20. Collado, J. A., Alvarez, I., Ciudad, M. T., Espinosa, G., Canals, F., Pujol-Rujau, P. A., Carrascal, M., Abian, J., and Jaraquemada, D. (2013) Combinatorial peptide presentation by human thymic epithelial cells reveals strong effects of protein abundance and turnover on antigen presentation. J. Immunol. 184, 1153–1157

21. van Haren, S. D., Herczenik, E., ten Brinke, A., Mertens, K., Voorberg, J., Fissolo, N., Haag, S., de Graaf, K. L., Drews, O., Stevanovic, S., Ramnauth, N., Pahau, H., Lee, B. T., Ng, J., G Brunck, M. E., Hyde, C., Trouw, L. A., Dudek, N. L., Purcell, A. W., O’Sullivan, B. J., Connolly, J. E., Paul, K. S., Lé Cao, K. A., and Thomas, R. (2015) Citrullinated peptide dendritic cell immunotherapy in HLA risk genotype-positive rheumatoid arthritis patients. Sci. Transl. Med. 7, 290ra87

22. van Haren, S. D., Herczenik, E., ten Brinke, A., Mertens, K., Voorberg, J., Fissolo, N., Haag, S., de Graaf, K. L., Drews, O., Stevanovic, S., Ramnauth, N., Pahau, H., Lee, B. T., Ng, J., G Brunck, M. E., Hyde, C., Trouw, L. A., Dudek, N. L., Purcell, A. W., O’Sullivan, B. J., Connolly, J. E., Paul, K. S., Lé Cao, K. A., and Thomas, R. (2015) Citrullinated peptide dendritic cell immunotherapy in HLA risk genotype-positive rheumatoid arthritis patients. Sci. Transl. Med. 7, 290ra87
teomics 10, M110.002477
45. Alvarez, I., Collado, J., Daura, X., Colomé, N., Rodríguez-Garcia, M., Gal- lart, T., Canals, F., and 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 (DRB4*0041). Arthritis Rheum. 58, 1630–1639
46. Frese, C. K., Attelaar, A. F., Herrmann, M. L., Notting, D., Zeller, M., Griepp- Raming, J., Heck, A. J., and Mohammed, S. (2011) Improved peptide identification by targeted fragmentation using CID, HCD and ETD on an LTQ-Orbitrap Velos. J. Proteome Res. 10, 2377–2388
47. Mommen, G. P. M., Frese, C. K., Meiring, H. D., van Gaans-van den Brink, J., de Jong, A. P. J. M., van Els, C. a. C. M., and Heck, A. J. R. (2015) Expanding the detectable HLA peptide repertoire using electron-transfer/higher-energy collision dissociation (EthCD). Proc. Natl. Acad. Sci. U.S.A. 112, 4507–12
48. Marino, F., Bern, M., Mommen, G. P. M., Leney, A. C., van Gaans-van den Brink, J. a., Bonvin, A. M. J. J., Becker, C., van Els, C. a. C. M., and Heck, A. J. R. (2015) Extended O-GlCNAC on HLA Class-I-Bound Peptides. J. Am. Chem. Soc., 150819112650001
49. Milner, E., Barnea, E., Beer, I., and Admon, A. (2006) The Turnover Kinetics of Major Histocompatibility Complex Peptide Antigens of Human Cancer Cells. Mol. Cell. Proteomics, 357–365
50. Weinzierl, A. O., Lemmel, C., Schoor, O., Muller, M., Kruger, T., Wernet, D., Hennenlotter, J., Stenzl, A., Klingel, K., Rammensee, H.-G., and Stevanovic, S. (2006) Distorted Relation between mRNA Copy Number and Processed MHC class II ligands. Mol. Immunol. 43, 567–575
51. Fortier, M.-H., Caron, É., Hardy, M.-P., Voisin, G., Lemieux, S., Perreault, C., and Thibault, P. (2008) The MHC class I peptide repertoire is molded by the transcriptome. J. Exp. Med. 205, 595–610
52. Blanco, P., Palacka, A. K., Pascual, V., and Banchereau, J. (2008) Dendritic cells and cytokines in human inflammatory and autoimmune diseases. Cytokine Growth Factor Rev. 19, 41–52
53. Watts, C. (2012) The endosome-lysosome pathway and information generation in the immune system. Biochim. Biophys. Acta 1824, 14–21
54. Chicz, R. M., Urban, R. G., Gorga, J. C., Vignali, D. A., Lane, W. S., and Strominger, J. L. (1993) Specificity and promiscuity among naturally processed peptides bound to HLA-DR alleles. J. Exp. Med. 178, 27–47
55. Rotzschke, O., and Falk, K. (1994) Origin, structure and motifs of naturally processed MHC class II ligands. Curr. Opin. Immunol. 6, 45–51
56. Juncker, A. S., Larsen, M. V., Weinhold, N., Nielsen, M., Brunak, S., and Lund, O. (2009) Systematic characterisation of cellular localisation and expression profiles of proteins containing MHC ligands. PLoS ONE 4, e7448
57. Schmid, D., Dengjel, J., Schoor, O., Stevanovic, S., and Münz, C. (2006) Autophagy in innate and adaptive immunity against intracellular pathogens. J. Mol. Med. 84, 194–202
58. Jaraquemada, D., Marti, M., and Long, E. O. (1990) An endogenous processing pathway in vaccinia virus-infected cells for presentation of cytoplastic antigens to class II-restricted T cells. J. Exp. Med. 172, 947–954
59. Jacobson, S., Sekaly, R. P., Jacobson, C. L., McFarland, H. F., and Long, E. O. (1989) HLA class II-restricted presentation of cytoplastic measles virus antigens to cytotoxic T cells. J. Virol. 63, 1756–1762
60. Liu, Y., Wang, L. X., Yang, G., Hao, F., Urba, W. J., and Hu, H. M. (2008) Efficient cross-presentation depends on autophagy in tumor cells. Cancer Res. 68, 6889–6895
61. Liu, Y., Wang, L. X., Pang, P., Cui, Z., Aung, S., Haley, D., Fox, B. A., Urba, W. J., and Hu, H. M. (2011) Tumor-derived autophagosome vaccine: mechanism of cross-presentation and therapeutic efficacy. Clin. Cancer Res. 17, 7047–7057
62. Wang, R. F., Wang, X., Atwood, A. C., Topalian, S. L., and Rosenberg, S. A. (1999) Cloning genes encoding MHC class II-restricted antigens: mutated CDC27 as a tumor antigen. Science 284, 1351–1354
63. Dengjel, J., Schoor, O., Fischer, R., Reich, M., Kraus, M., Müller, Kreyborg, K., Altenberend, F., Brandenburg, J., Käßbauer, H., Brock, R., Diessen, C., Rammensee, H. G., and Stevanovic, S. (2005) Autophagy promotes MHC class II presentation of peptides from intracellular source proteins. Proc. Natl. Acad. Sci. U.S.A. 102, 7922–7927
64. Münz, C. (2009) Enhancing immunity through autophagy. Annu. Rev. Immu- nol. 27, 423–449
65. Crotzer, V. L., and Blum, J. S. (2009) Autophagy and its role in MHC- mediated antigen presentation. J. Immunol. 182, 3335–3341
66. Herberts, C. a., Meiring, H. M., Van Gaans-van den Brink, J. a. M., Van der Hee, E., Poelen, M. C. M., Boog, C. J. P., De Jong, A. P. J. M., and Van Els, C. a. C. M. (2003) Dynamics of measles virus protein expression are reflected in the MHC class I epitope display. Mol. Immunol. 39, 567–575
67. Bozzacco, L., Yu, H., Zebroski, H. A., Dengjel, J., Deng, H., Mojsov, S., and 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
68. Yewdell, J. W. (2006) Confronting complexity: real-world immunodomi- nance in antiviral CD8+ T cell responses. Immunity 25, 533–543
69. Blum, J. S., Wearsch, P. A., Cresswell, P. (2013) Pathways of antigen processing. Annu Rev Immunol. 31, 443–473
70. Villadangos, J. A., Schnorrer, P., and Wilson, N. S. (2005) Control of MHC class II antigen presentation in dendritic cells: a balance between creative and destructive forces. Immunol. Rev. 207, 191–205
71. Novellino, L., Renkvist, N., Rini, F., Mazzocchi, A., Rivoltini, L., Greco, A., Deho, P., Squarciuna, P., Robbins, P. F., Parmiani, G., and Castelli, C. (2003) Identification of a mutated receptor-like protein tyrosine phosphatase kappa as a novel, class II HLA-restricted melanoma antigen. J. Im- munol. 170, 6363–6370
72. Syka, J. E., Coon, J. J., Schroeder, M. J., Shabanowitz, J., and Hunt, D. F. (2004) Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. Proc. Natl. Acad. Sci. U.S.A. 101, 9528–9533

Molecular & Cellular Proteomics 15.4