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Mass Tag-Assisted Identification of Naturally Processed HLA Class II-Presented Meningococcal Peptides Recognized by CD4⁺ T Lymphocytes

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The meningococcal class I outer membrane protein porin A plays an important role in the development of T cell-dependent protective immunity against meningococcal serogroup B infection and is therefore a major component of candidate meningococcal vaccines. T cell epitopes from porin A are poorly characterized because of weak in vitro memory T cell responses against purified Ag and strain variation. We applied a novel strategy to identify relevant naturally processed and MHC class II-presented porin A epitopes, based on stable isotope labeling of Ag. Human immature HLA-DR1-positive dendritic cells were used for optimal uptake and MHC class II processing of ¹⁴N- and ¹⁵N-labeled isofoms of the neisserial porin A serosubtype P1.5–2,10 in bacterial outer membrane vesicles. HLA-DR1 bound peptides, obtained after 48 h of Ag processing, contained typical spectral doublets in mass spectrometry that could easily be assigned to four porin A regions, expressed at diverging densities (~30–4000 copies/per cell). Epitopes from two of these regions are recognized by HLA-DR1-restricted CD4⁺ T cell lines and are conserved among different serosubtypes of meningococcal porin A. This mass tag-assisted approach provides a useful methodology for rapid identification of MHC class II presented bacterial CD4⁺ T cell epitopes relevant for vaccine development. The Journal of Immunology, 2005, 174: 5636–5643.

Infections by Neisseria meningitidis serogroup B are the most common cause of bacterial meningitis or sepsis in the western world (1). The natural habitat of the meningococcus is the nasopharynx, and meningococcal carriage, a naturally immunizing status, occurs in approximately 10% of the population. The development of meningococcal disease depends on poorly defined environmental, microbial, and (immuno)genetic factors. The rapid increase in the incidence of meningococcal disease, with high morbidity and mortality in previously healthy young infants and adolescents, urges the need for a vaccine (2). The polysaccharide of serogroup B isolates is poorly immunogenic, and alternative vaccine candidates, including outer membrane proteins, are under investigation. High affinity bactericidal Ab titers against the variable major outer membrane protein porin A (PorA)² are thought to protect against invasion of the meningococcus and disease. Preclinical studies using candidate serogroup B meningococcal vaccines based on outer membrane vesicles (OMV) from genetically manipulated meningococci point out that different PorA wild types may not be equally immunogenic (3–5). Since the induction and maintenance of anti-PorA Ab titers are typically T cell dependent, dissection of the MHC class II restricted CD4⁺ T cell response against this protein is paramount for further vaccine improvement. Although meningococcal outer membrane proteins, including PorA, have been shown to be immunogenic to human CD4⁺ T cells (6–8), only a few PorA-specific T cell epitopes were successfully mapped (9). T cells recognize naturally processed peptides presented by MHC molecules at the cell surface of professional APC (10, 11), and T cell epitopes from defined Ags may be predicted using MHC peptide binding algorithms (12, 13). However, particularly for CD4⁺ T cell epitopes, this approach has a high failure rate because the motifs for binding to MHC class II molecules are little confined (14, 15) and, moreover, because predicted epitopes may not be naturally processed in the cell. As an alternative, mass spectrometry (MS) provides for the direct analysis and identification of processed and presented candidate T cell epitopes, through sampling the plenitude of MHC-associated peptides isolated from Ag pulsed APC and comparing these with peptides obtained from unpulsed APC (16–23).

To explore the MHC presentation of PorA by MS but circumvent this laborious comparative approach, we here developed a strategy that tags vaccine components using stable isotope labeling for rapid nanoscale liquid chromatography electrospray ionization mass spectrometry (nLC-ESI-MS) detection of Ag-derived MHC class II epitopes in a single cellular extract of in vitro cultured human dendritic cells (DC). This approach led to the identification of various PorA regions that are naturally processed and presented by HLA-DR1 and are recognized by human CD4⁺ T cells.

Materials and Methods

Growth of neisserial P1.5–2,10 strain in minimal medium and OMV preparation

The construction of a class 3, class 4 isogenic H44/76 strain expressing the serosubtype P1.5–2,10 was described previously (24). For stable isotope protein labeling, the P1.5–2,10 strain was grown until stationary.
phase, either in natural $^{14}$NH$_4$Cl-containing minimal meningoococal medium, or in minimal meningoococal medium containing >98% enriched stable $^{15}$NH$_4$Cl (Spectra Stable Isotopes), respectively (25). From these cultures, batches of $^{14}$N- and $^{15}$N-OMV were prepared and characterized according to Claassen et al. (26) and subjected to MS analysis (27).

Isolation of PBMC

PBMC from HLA-oligotyped donors after leukapheresis were isolated by centrifugation of buffy coat cells on Ficoll-Hypaque (Pfizer) and were freshly used or cryopreserved until usage in experiments.

Culturing, Ag pulse, and characterization of DC

Human CD83$^+$, HLA-DR$^+$ immature DC (iDC) were cultured according to a procedure by Sallusto et al. (28). Briefly, $1 \times 10^5$ PBMC were freshly isolated from a HLA-DRB1*0101 homozygous donor and seeded at $5 \times 10^5$ PBMC/ml in 150-mm tissue culture dishes (Costar) in IMDM (Invitrogen Life Technologies) supplemented with 1% FCS at 37°C. 5% CO$_2$ in a humidified incubator, for 2 h. After removal of the non-adherent fraction, adherent cells were further cultured for 6 days in medium containing 500 U/ml recombinant human GM-CSF (PeproTech) and 250 U/ml recombinant human IL-4 (Strathman Biotech). Culture medium and growth factors were refreshed on day 3. On day 6, iDC were pulsed with a mixture of $^{14}$N-P1.5–2,10 OMV and $^{15}$N-P1.5–2,10 OMV (at a protein ratio of 1:1) at a total protein concentration of $\sim 0.02$ mg/ml and incubated for 6 h. Hereafter, cells were further cultured and matured in the continuous presence of IFN-$\gamma$ ($\sim 0.01$ mg of protein/ml), growth factors, and 20 $\mu$g/ml LPS (Salmonella abortus equi; Sigma-Aldrich). On day 8, OMV-pulsed mature DC (mDC) were harvested, washed in PBS containing 2.0 mN EDTA, counted, pelleted, snap frozen on dry ice, and held in $\sim 135^\circ$C. Typically the protocol yields $50–90 \times 10^6$ mDC from $1 \times 10^7$ PBMC. Small aliquots of immature and mature OMV-pulsed DC were characterized by flow cytometry, using FITC-conjugated or PE-conjugated anti-human mAbs specific for HLA-DR (Sigma clone HK14), CD80 (Serotec), CD86 (BD Pharmingen), CD83 (BD Pharmingen), CD40 (BD Pharmingen), and appropriate isotype matched controls. After staining samples were washed with cold FACS buffer and approximately 10,000 events were acquired in the FSC-SSC gate for large cells on a FACSCalibur flow cytometer, using FITC-conjugated or PE-conjugated anti-human mAbs specifically for HLA-DR (Sigma clone HK14), CD80 (Serotec), CD86 (BD Pharmingen), CD83 (BD Pharmingen), CD40 (BD Pharmingen), and appropriate isotype matched controls. After staining samples were washed with cold FACS buffer and approximately 10,000 events were acquired in the FSC-SSC gate for large cells on a FACSCalibur flow cytometer (BD Biosciences) and analyzed using CELLQuest software (BD Biosciences).

Peptide synthesis

Peptides for T cell studies were prepared by $N$-(9-fluorenylmethoxycarbonyl (Fmoc) solid phase synthesis in a SYRO II simultaneous multipeptide synthesizer (MultiSynTech). Heavy isotope-labeled variants of peptides for MS purposes were synthesized using (99 atom%) $N$-Fmoc-$^{13}$C$_2$-glycine at the $G^*$ position (Cambridge Isotope Laboratories).

HLA-DR-associated peptide isolation and nLC-ESI-MS

HLA-DRB1*0101 molecules were immunoprecipitated from $8 \times 10^6$ OMV-pulsed DC, essentially as described previously (22), using the HLA-DR-specific mAb B8.11.2 bound to cyanogen bromide (CNBr)-activated Sepharose 4B beads. Peptides were eluted using 10% acetic acid, and spun through a 10-kDa cutoff spinfilter (Millipore). Peptide analysis of samples equivalent to $1–2 \times 10^6$ DC was performed using nLC-ESI-MS (27) on a quadrupole ion trap instrument LCQ Classic (Thermo Finnigan MAT) and a QTOF-Ultima instrument (Micromass) at a resolution of 11,000. Mass spectra were searched for characteristic $^{15}$N$^{15}$N/ $^{14}$N$^{14}$N doublet formation of OMV-derived peptides, using an in-house-developed algorithm based on MATLAB software (MathWorks). The algorithm can distinguish the mass spectral information belonging to $^{15}$N$^{15}$N/ $^{14}$N$^{14}$N ion doublets from the complex background of mass spectra from self-peptides at very high sensitivity (noise level), due to 1) the typical relative mass difference of 1.2% between the $^{14}$N and $^{15}$N ions, 2) their one-to-one ratio, and 3) the characteristic isotope pattern of the $^{15}$N isomers (due to 98 atom% labeling). Candidate OMV-derived $^{15}$N ions were selected for identification by MS/MS sequencing using collision-induced dissociation energy. The sequences of the allocated peptides were confirmed using synthetic peptides. For quantitation of the amount of a peptide in a sample, peptide mixtures were spiked with known amounts of two synthetic peptide standards angiotensin II and oxytocin (Sigma-Aldrich) directly after peptide isolation to correct for sample loss during the subsequent processing of the samples, assuming equal specific responses (counts per picomole) and equal losses for peptides and internal synthetic standards.

Generation of PorA-specific CD4$^+$ T cell lines (TCL)

PorA-specific TCL were generated from HLA-DRB1*0101 homozygous adult donors after culturing of PBMC ($2 \times 10^5$ cells/well) in 96-well round-bottom plates in AIM-V medium in the presence of 2% human serum and 2–20 $\mu$M synthetic peptide or 0.5 $\mu$g/ml recombinant PorA (rPorA). Ten days after the initiation of the cultures, 10 U/ml rL-2 (Cetus) was added. TCL were repetitively restimulated at 14- to 21-day intervals, using $2 \times 10^5$ peptide- or rPorA-pulsed, irradiated autologous or HLA-DR matched PBMC and rL-2.

T cell proliferation assay

Proliferation assays were performed as described by Van Bleek et al. (29). Briefly, either PBMC ($10^5$/well) or TCL ($2 \times 10^5$/well, supplemented with $10^5$ irradiated autologous PBMC/well) from HLA-DRB1*0101-positive individuals were cultured in 96-well round-bottom microculture plates in AIM-V medium (Invitrogen Life Technologies) containing 2% human serum and in the presence of either no Ag, synthetic peptide, or recombinant P1.5–2,10 (rP1.5–2,10) (or other PorA serotypes, generously provided by G. Zlotnick, Wyeth Vaccines) at indicated doses. In blocking experiments, irradiated PBMC were preincubated in the presence or absence of culture supernatant (1:100) from the HLA-DR-specific hybridoma B8.11.2 or from the HLA-DQ-specific hybridoma SPV-L3 before addition of Ag and coculture with TCL. After 5 days (for PBMC) or 2 days (for TCL) the cultures were pulsed with 0.5 $\mu$Ci of [$^{3}$H]thymidine (Amersham Biosciences). Cultures were harvested 16 h later on filters using a 96-well sample harvester (LKB Wallac), and cpm were determined using a liquid scintillation beta counter (LKB Wallac). Results obtained from triplicate or six replicate cultures were represented as stimulation index as follows: stimulation index = (mean cpm obtained in the presence of APCs and Ag)/(mean cpm obtained in the presence of APCs and medium only).

ELISPOT assay

IFN-$\gamma$ ELISPOT assays were performed according to Van Bleek et al. (29). Briefly, 96-well filtration plates (MAIP5450; Millipore) were coated overnight with anti-IFN-$\gamma$ coating Ab (clone 1-D1K, Mabtech) in carbonate buffer at 4°C. After washing and blocking of the plates, 2 $\times 10^5$ PBMC or 2 $\times 10^5$ TCL per well were seeded in culture medium in the presence or absence of Ag, as indicated, and for TCL supplemented with 2 $\times 10^5$ irradiated autologous or HLA-DR matched PBMC, for a 24 h incubation at $37^\circ$C, 5% CO$_2$, in a humidified incubator. Then, plates were washed, developed with chromogenic alkaline phosphate substrates after a two-step incubation with biotinylated anti-IFN-$\gamma$ detecting Ab (clone 7-B6-1; Mabtech) and streptavidin-alkaline phosphatase, and the number of spot-forming cells (SFC) per well was counted using AELVIS hardware and software (AELVIS). Responses were considered positive if the number of SFC/well was five or more and at least twice that in wells containing medium or control Ag.

Results

Preparation of $^{14}$N- and $^{15}$N-labeled OMV from a neisserial isogenic strain expressing P1.5–2,10

PorA mounts to approximately 90% of the protein content of experimental OMV-based vaccines isolated from heat-inactivated meningococci after deoxycholate extraction and vesicle purification (26). To allow synthesis of stable light and heavy forms of meningoococcal outer membrane proteins, 1 L log-phase cultures of the class 3-, class 4- isogenic H44/76 strain expressing the PorA serosubtype P1.5–2,10 were prepared in $^{14}$N-containing medium, or in 98% $^{15}$N-enriched medium, respectively. OMV prepared from these cultures contained 2 and 1.7 mg of protein/ml, respectively, and had identical PorA migration patterns in SDS-PAGE (data not shown). nLC-ESI-MS analysis of the contents of $^{14}$N- and $^{15}$N-OMV identified a mass increment (1.2%) for the heavy form of the P1.5–2,10 protein relative to its light form (Fig. 1). In addition, MS/MS spectra obtained from trypsin digestion products from $^{14}$N- and $^{15}$N-OMV revealed typical fragmentation into heavy and light amino acids, respectively (data not shown), confirming the successful stable isotope labeling throughout the full sequence of the P1.5–2,10 protein.
OMV-pulsed DC, bound peptides were eluted and separated by nLC-MS analysis as described (27). The measured average m.w. of each protein closely approximates the expected values. A relative increase of 1.2% is observed indicating a uniform 15N-labeling of the protein.

Loading of cultured human HLA-DR1 homozygous DC with stable isotope-labeled P1.5–2,10 OMV

To study Ag presentation relevant for T cell priming we applied an in vitro culture system for the outgrowth of human monocyte-derived immature DC for Ag uptake and processing (28). Adherent PBMC obtained from a homozygous HLA-DRB1*0101 typed donor were cultured in medium supplemented with human growth factors. After 6 days, DC are still immature and appear as loosely adherent clumps or as isolated floating cells with typical dendritic morphology (data not shown). At this stage, a 1:1 (protein ratio) mixture of 14N- and 15N-labeled P1.5–2,10 OMV was fed to the cultures, and Ag uptake, processing, and maturation by DC were allowed for 48 h in the presence of growth factors and LPS. At day 8, loosely adherent and floating DC were collected and washed extensively before MHC peptide isolation. Maturation of DC under these conditions was illustrated by the upregulation of DC marker CD83 and the co-stimulatory molecules CD80 and CD86, whereas expression of HLA-DR and CD86 remained relatively unchanged (Fig. 2). These phenotype changes are similar to those described for clinical grade DC (30).

Mass tag-assisted identification of naturally processed and presented epitopes derived from four regions of the P1.5–2,10 protein, which were expressed at diverging estimated densities, i.e., 30–4000 copies per cell (Table I; Fig. 4). To find evidence for the existence of additional members of nested set regions I, II, and IV that might have been missed by our algorithm, mass spectra were searched manually for the presence of low abundant 14N/15N ion doublets representing N- or C-terminally extended or shortened length variants. At the applied detection limit of the MS system, i.e., approximately three copies per cell, none were detected.

CD4+ T cell reactivity to PorA91–108 and PorA349–367 in HLA-DR1-positive individuals

To assess whether the four naturally processed P1.5–2,10 regions represent human meningococcal CD4+ T cell epitopes, PBMC from three HLA-DR1 homozygous healthy blood donors were tested ex vivo in a [3H]thymidine proliferation assay using overlapping synthetic peptides (18 aa) encompassing these sequences. Direct specific proliferation was measured against all four regions in at least two of three donors tested (summarized in Table I). Repetitive in vitro stimulation of HLA-DR1 homozygous PBMC with peptides representing P1.5–2,10349–367, but not P1.5–2,10349–367, led to the outgrowth of epitope-specific CD4+ T cell lines, also recognizing autologous rP1.5–2,10-pulsed APC (data not shown). Further support for the dominant immunogenicity of the P1.5–2,10349–367 and P1.5–2,10349–367 regions came from studies using a CD4+ bulk culture.
established from PBMC from an HLA-DR1 homozygous donor after 3–5 repetitive in vitro restimulations with autologous rP1.5–2,10-pulsed APC, allowing any rP1.5–2,10 derived HLA-DR1 epitopes to be presented at naturally processed levels. 410/5–2,10 CD4 T cells proliferated specifically in response to rP1.5–2,10 (data not shown) and showed fine specificity uniquely for the P1.5–2,1091–108 and P1.5–2,10349 –367 regions when tested against a full protein pepscan in IFN-ELISPOT (Fig. 5).

Table I. Naturally processed and HLA-DR1-presented epitopes derived from P1.5–2,10

<table>
<thead>
<tr>
<th>PorA Sequence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Peptide Sequence&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Abundance&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Proliferation&lt;sup&gt;d&lt;/sup&gt;</th>
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<tr>
<td>P1.5–2,10&lt;sub&gt;-18&lt;/sub&gt;</td>
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<td>DVSLYGEGIKAVGVEGRNILQ</td>
<td>1660</td>
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<tr>
<td>P1.5–2,10&lt;sub&gt;91–108&lt;/sub&gt;</td>
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<td>750</td>
<td>3/3</td>
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<tr>
<td>P1.5–2,10&lt;sub&gt;92–106&lt;/sub&gt;</td>
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<td>290</td>
<td></td>
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<tr>
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<td>GEFGTLRAGRVANQ</td>
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<td></td>
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<tr>
<td>P1.5–2,10&lt;sub&gt;94–106&lt;/sub&gt;</td>
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</tr>
<tr>
<td>P1.5–2,10&lt;sub&gt;98–364&lt;/sub&gt;</td>
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<td>1810</td>
<td>2/3</td>
</tr>
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<td>3/3</td>
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<td>P1.5–2,10&lt;sub&gt;105–364&lt;/sub&gt;</td>
<td>GGYGGYQINASVGLR</td>
<td>290</td>
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</table>

<sup>a</sup>PorA residue numbering according to the appearance in the protein chain after cleavage of the N-terminal signal peptide.

<sup>b</sup>Confirmed by MS/MS sequencing.

<sup>c</sup>Estimated copy number per cell.

<sup>d</sup>Number of HLA-DR1 homozygous PBMC donors out of number tested reactive against synthetic peptides encompassing the PorA region in a direct proliferation assay.

<sup>e</sup>Potential anchor residue.

FIGURE 3. Mass tag-assisted identification of a naturally processed P1.5–2,10 peptide in HLA-DRB1*0101. Peptides were isolated from in vitro cultured human HLA-DRB1*0101-positive DC after pulsing with 14N- and 15N-OMV (at a ratio of 1:1) derived from the isogenic neisserial strain expressing P1.5–2,10 as described in Material and Methods. Top panel, the ESI mass spectrum obtained at a retention time of 26.5 min containing a triply charged mass spectral doublet at m/z 553.97 and 561.94 atomic mass unit; inset, the deconvoluted MH spectrum indicating a putative neisserial peptide containing 24 nitrogen atoms; middle panel, the deconvoluted MS/MS spectrum of this peptide at m/z 553.97 atomic mass unit revealing a partial sequence (y-ions series, y11-y15) of the P1.5–2,10 originating peptide LAGEFGTLRAGRVANQ; bottom panel, the collision-induced dissociation mass spectra of its isotopically labeled synthetic analog, prepared using N-Fmoc-2-13C-glycine at the G* position (LAGEFG*TRTAG*RVANQ).
To elaborate on the molecular requirements of presentation and recognition of one of the immunodominant epitope regions, a CD4⁺ T cell clone (JS20) specific for synthetic P1.5–2,1091–108 peptide (Fig. 6a) was established and functionally tested in more detail. JS20 T cells responded to the two most abundant length variants, P1.5–2,1091–106 and P1.5–2,1092–106, with comparable dose-response rates (Fig. 6b). To confirm that specific P1.5–2,10 recognition by JS20 T cells occurred after natural Ag processing in the context of HLA-DR1, autologous HLA-DR1-matched or -mismatched PBMC were allowed to process rP1.5–2,10 for a minimum of 2 h before irradiation and co-culturing for 48 h with JS20 T cells. Proliferation of JS20 T cells was induced by processed rPorA in a dose-dependent manner (Fig. 6c) required the presence of the HLA-DR1 allele, and was inhibitable by the presence of HLA-DR blocking mAbs (Fig. 6d). Finally, the effects of N- and C-terminal truncations and single amino acid substitutions within the P1.5–2,1091–106 sequence on JS20 T cell recognition were investigated (data summarized in Table II). A minimal core length of 11 aa (P1.5–2,1094 –104) was required for preservation of JS20 T cell proliferation or IFN-γ production. In this sequence, alanine or charge substitutions at positions E94,F 95,T 97,L 98,R 99,G 101,R 102, and V103 clearly diminished or abolished the ability to stimulate JS20 T cell activity. From these analyses hypothetical contact residues important for the fit of the P1.5–2,1091–106 epitope into the HLA-DR1 binding groove and for interaction with the JS20 TCR can be proposed (Fig. 7).

Discussion

To unravel which and how many antigenic determinants are involved in the induction of CD4⁺ T cell responses against a pathogen is an important objective in rational vaccine design. In this study, two major advances were made toward improved direct identification of naturally processed CD4⁺ T cell epitopes derived from a vaccine candidate. First, we employed the efficient processing and presentation machinery of in vitro cultured immature human DC to load vaccine components onto MHC class II molecules. Second, in analogy with their use in proteomics (31), stable isotopes were applied during Ag synthesis to guide the MS identification of Ag-derived MHC class II-presented epitopes in a single peptide preparation through typical ion doublet formation. This method appears highly efficient, since multiple PorA-derived HLA-DR1-associated epitopes could be identified using samples equivalent to as few as 1–20 × 10⁶ DC. To our knowledge, this is the first study using human DC for Ag uptake and MS epitope identification.
identification. Others who previously succeeded in MS characterization of disease or Ag-specific HLA class II ligands employed relatively large quantities of EBV-transformed lymphoblastoid B cells (19, 20, 32, 33), or spleen cells (34), not being necessarily the relevant APC for T cell priming (35). By contrast, DC are important in vivo priming APC. They efficiently capture Ags while in an immature state and acquire the ability to optimally process Ag and stably present antigenic MHC class II peptide complexes upon maturation (36, 37).

Neisserial P1.5–2,10 epitopes identified through mass tagging appeared to be derived from four regions of the protein (Fig. 4) and were estimated at expressed densities ranging from 30 copies per cell (P1.5–2,1091–108) to 3680 copies per cell (P1.5–2,10353–367). These levels of expression were comparable to those found for self-epitopes in the HLA-DR eluate (the most abundant of which was expressed at 2200 copies per cell, data not shown), indicating that sufficient exogenous P1.5–2,10 Ag was taken up and processed by DC to compete for HLA-DR loading. Typical of MHC class II in DC, our method guided and simplified further CD4+ T cell recognition. They efficiently capture Ags while in an immature state and acquire the ability to optimally process Ag and stably present antigenic MHC class II peptide complexes upon maturation (36, 37).

Table II. Effect of N- and C-terminal truncation and alanine substitution of P1.5–2,1091–106 on JS20 T cell recognition

<table>
<thead>
<tr>
<th>Peptide P1.5–2,1091–106</th>
<th>Truncation Variants</th>
<th>JS20a</th>
<th>Alanine Substitution</th>
<th>JS20</th>
<th>Charge Substitution</th>
<th>JS20</th>
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<tr>
<td>LAGEFGTLRAGVRANQ</td>
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* Shown is the relative responsiveness of JS20 CD4+ T cells in a proliferative assay in the presence of HLA-DRB1-positive PBMC and synthetic truncation and alanine substitution variants of the naturally occurring P1.5–2,1091–108 epitope at 1 μM.

Counts per minute in the presence of medium or P1.5–2,1091–108 were 137 and 41,057, respectively. Identical trends were observed in IFN-γ ELISPOT (data not shown). A dose response curve for the F95A variant is also seen in Fig. 6b.

The topology model of outer membrane porins in Neisseria meningitidis (39) predicts eight surface-exposed hydrophilic loops, connected by membrane-traversing β strands and internal turns, consistent with the β barrel structure of outer membrane proteins of bacterial origin (40). According to this model, the four HLA-DRB1*0101-presented PorA regions are all membrane-spanning sequences, located either toward the N- or the C-terminal end of the porin, in fact having its exact N and C termini (Fig. 4). The HLA-DRB1*0101 binding groove accommodates a bulky hydrophobic P1 peptide anchor residue and smaller less hydrophobic P4, P6, P7, and/or P9 peptide anchors residues (41). Due to their hydrophobic nature all identified epitope regions comply to this motif and could in fact have been predicted as HLA-DRB1*0101 binding epitopes, together with a greater variety of candidate epitopes from other membrane-spanning domains of the P1.5–2,10 molecule. However, no other regions appear to be successfully processed and presented. The rules dictating crypticity and dominance or subdominance of epitopes are not fully understood (42). One requirement for epitope expression is the activity of multiple proteases in the MHC class II pathway (10), and there is emerging evidence that individual enzymes can make distinct and non-redundant contributions to the generation (or destruction) of epitopes from a particular protein Ag (43–46). Two observations suggest that processing of the P1.5–2,10 protein in DC occurs in a selective manner. First, no epitopes derived from the more central region of the P1.5–2,10 molecule were found in the HLA-DRB1*0101 eluate, despite the overall presence of stretches of hydrophobic amino acids containing the DR1 binding motif. Second, inspection of the ten eluted and sequenced epitopes revealed a non-random C terminus. Whereas the P1.5–2,1091–367 epitope contains the native C-terminal F967 of the P1.5–2,10 molecule, seven of the nine other epitopes, derived from three different regions of the P1.5–2,10 molecule, terminated at Q or R (Table I). A similar C-terminal preference was found for multiple self-epitopes displayed by HLA-DRB1*0401 molecules extracted from EBV-transformed lymphoblastoid B cells (38), pointing at a shared selective protease activity in the MHC class II pathway of different human APCs operating at a variety of protein substrates.

By unambiguously denominating which regions of the P1.5–2,10 Ag become successfully processed and presented by the MHC class II in DC, our method guided and simplified further CD4+ T cell immunogenicity studies. Ex vivo T cell reactivity against peptides representing all four identified P1.5–2,10 regions was observed in healthy HLA-DRB1*0101-positive individuals (Table I), confirming indeed that epitopes expressed after in vitro processing of OMV by human DC are relevant for CD4+ T cell priming after natural exposure to meningococci. However, stable CD4+ T cell lines and clones could only be established against the immunodominant P1.5–2,1091–108 and P1.5–2,1091–367 regions. Notably, from both these regions, most members of nested sets were found,
including peptide species expressed at a relatively moderate level (≤300 copies per cell). Also, both regions are conserved between PorA serotypes. It remains to be seen whether other PorA-derived CD4+ T cell epitopes associated with additional HLA class II haplotypes are sequence-conserved as well. If so, differences in immunogenicity between PorA serotypes cannot be accounted for by the absence or presence of strain unique T cell epitopes, but could still relate to differences in processing efficiency. Preliminary data with the P1.5-2,1091–104 epitope indicate that this latter possibility might indeed be the case. HLA-DR1-positive PBMC pulsed with rPorA serotypes other than P1.5-2,10 are also recognized by J520 T cells but at diverging dose-response rates (C. van Els, unpublished data). Further studies to elucidate the effect of PorA strain variation on the efficiency of processing and HLA-DR presentation are ongoing.

Recognition of the immunodominant P1.5-2,1091–104 region was studied in more detail using the J520 CD4+ T cell clone. A shared core sequence, P1.5-2,1091–104, was minimally required for HLA-DR1*0101 presentation and J520 recognition, whereby 

\[ \text{F}_{90} \]  was proposed to serve as the P1 anchor residue (Table II; Fig. 7). These data also suggested that the predominance of Q106 as the C-terminal residue of the natural length variants of this region is not related to its critical requirement for binding but rather the result of selective epitope trimming (47). Additional work has indicated that the P1.5-2,1091–104 region is also immunogenic in the context of the closely related HLA-DRB1*1001 allele (C. van Els, unpublished data). Hence, being efficiently processed, antigenically conserved, and presented in the context of various HLA-DR alleles, the P1.5-2,1091–104 region is an immunologically important target in neisserial CD4+ T cell immunity.

Finally, metabolic stable isotope-labeling is widely applicable to protein expression systems or pathogen cultures. Therefore, our target in neisserial CD4+ T cell immunity.

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Disclosures

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