Differential Binding of Pyruvate Dehydrogenase Complex-E2 Epitopes by DRB1*08:01 and DRB1*11:01 Is Predicted by Their Structural Motifs and Correlates with Disease Risk

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Differential Binding of Pyruvate Dehydrogenase Complex-E2 Epitopes by DRB1*08:01 and DRB1*11:01 Is Predicted by Their Structural Motifs and Correlates with Disease Risk

I-Ting Chow,*1 Eddie A. James,*1 Theresa J. Gates,* Venus Tan,* Antonis K. Moustakas,† George K. Papadopoulos,‡ and William W. Kwok*§

Alleles DRB1*08:01 (DR0801) and DRB1*11:01 (DR1101) are highly homologous and differ by only 6 aa residues (1). In addition to reports of its influence on susceptibility to juvenile arthritis (2, 3), DR0801 is the most consistently reported genetic association with primary biliary cirrhosis (PBC), a chronic autoimmune liver disease for which the diagnostic criteria include anti-mitochondrial Ab positivity (4–6). In contrast, the highly homologous DR1101 allele was reported to protect against PBC (4, 5). Among diverse anti-mitochondrial Ab determinants, 90–95% of se-

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1T.C. and E.A.J. contributed equally to this work.

Address correspondence and reprint requests to Dr. William W. Kwok, Benaroya Research Institute at Virginia Mason, 1201 9th Avenue, Seattle, WA 98101-2795. E-mail address: bkwok@benaroyaresearch.org

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rum samples from patients with PBC react against the E2 subunit of pyruvate dehydrogenase complex (PDC-E2), making this the most important autoantigen in the disease (7). PBC is linked with multiple environmental factors that are thought to play a role in disease, including infection by viruses, such as the human β retrovirus, which was shown to cause aberrant expression of mitochondrial proteins on the biliary epithelium (8). Ectopic expression of proteins, such as PDC-E2, along with accompanying immunologic danger signals, could activate the autoreactive T and B cells that appear to potentiate the disease.

Diverse HLA class II molecules are differentiatated by polymorphic residues, mainly found within their peptide-binding pockets. As such, each class II allele binds and presents a distinct subset of peptides. It has been widely hypothesized that the role of self-HLA recognition in T cell–repertoire development may account for the influence of HLA genotype on autoimmune disease susceptibility (9). DR0801 shares a key structural feature with other HLA class II alleles that predispose to autoimmunity, lacking a canonical aspartic acid residue at position beta57 (10). This residue is reported to influence both the peptide-binding repertoire and the biochemical stability of HLA class II proteins (11–14). An aspartic acid at this position allows the formation of a salt bridge with the α76 arginine residue, and two consequent hydrogen bonds stabilize the αβ-heterodimer–peptide complex. Alleles that lack this negatively charged residue are unable to form this salt bridge, often relying on negatively charged peptide ligands or TCRs with negatively charged CDR3 regions to stabilize that positive charge (10, 15). This may also lead to important differences in HLA–peptide complex stability, increasing the number or avidity of autoreactive T cells.

Based on the molecular characteristics of DR0801 and its known association with PBC, we hypothesized that its peptide-binding
preferences would be distinct from DR1101 (which is homologous but protective), favoring the presentation of unique self-peptides and selecting a potentially autoreactive T cell repertoire. We addressed this question first by defining and comparing DR0801- and DR1101-restricted T cell epitopes within common vaccine Ags. We then characterized the peptide-binding motif of DR0801, measuring binding affinities for peptides with one or more amino acid substitutions and confirming key features of the DR0801 motif using structural modeling. Based on these findings, we predicted T cell epitopes within PDC-E2, assessed the binding of these peptides to DR0801 and DR1101, and evaluated the antigenicity of these sequences in subjects with DR0801 haplotypes.

Materials and Methods

Peptides, DR0801 protein, and DR1101 protein

Peptides with sequences derived from Influenza A/New Caledonia/2099 Hemagglutinin (H3HA), Influenza A/Wisconsin/67/2005 Hemagglutinin (H3HA), Influenza A/Puerto Rico/8/34 Matrix Protein (H1MP), Influenza A/Puerto Rico/8/34 Nucleoprotein (H1NP), and tetanus toxoid L chain (TTL), or tetanus toxin H chain (TT) were synthesized by Sigma (PEScreen; Sigma, St. Louis, MO). In addition, biotinylated H1MP98–110 (KLYRLKREITHF) and HA96–118 (PKYVKQNTLKLAT) peptide was synthesized by Cys-Gen. Peptides were dissolved in DMSO at 20 mg/ml and diluted as needed. Recombinant DR0801 and DR1101 proteins were produced essentially as described previously (16), with the exception that, for DR0801, the picornavirus-like 2A peptide was used to express the DR α- and β-chains as a single transcript to improve expression. The extracellular DR8α domain (plus a basic leucine zipper) was linked to the extracellular DR8β domain (plus an acidic leucine zipper) by the P2A peptide using splicing by overlapping-extension PCR. The resulting product was verified by sequencing with the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, Carlsbad CA) and subcloned into metallothionein-inducible pMT/V5-His A vector (Invitrogen, Grand Island NY). DR0801 and DR1101 were purified from insect cell culture supernatants by affinity chromatography and dialyzed against phosphate storage buffer (pH 6).

DR0801 and DR1101 tetramers

DR0801 or DR1101 protein was biotinylated at a sequence-specific site using biotin ligase (Avidiit, Denver, CO) and dialyzed into phosphate storage buffer. The biotinylated monomer was loaded with 0.2 mg/ml peptide by incubating at 37°C for 72 h in the presence of 2.5 mg/ml n-octyl-β-D-glucopyranoside and 1 mM Pefabloc SC protease inhibitor mix (Sigma-Aldrich). Peptide-loaded monomers were subsequently conjugated as tetramers using R-PE streptavidin (Biosource International, Camarillo, CA) at a molar ratio of 8:1.

Human subjects

Healthy subjects with DR0801 haplotypes or DR1101 haplotypes were recruited with written consent as part of an Institutional Review Board-approved study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Each subject was previously vaccinated against influenza and tetanus and had no history of autoimmune disease.

Tetramer-guided epitope mapping

The tetramer-guided epitope-mapping procedure was conducted as previously described (17) for each protein (H1HA, H3HA, H1MP, H1NP, TTL, and TT). Briefly, PBMCs were isolated from the blood of vaccinated healthy DR0801 or DR1101 subjects by Ficoll underlay, and CD4+ T cells were purified by negative selection using an isolation kit (Miltenyi Biotec, Auburn, CA). Adherent cells from the CD4+ fraction were used as APCs by incubating with 3 × 10⁶ cells/well in 300 μl volume in 48-well plates for 1 h and washing. Two million CD4+ T cells/well were stimulated with pools of five consecutive peptides. After 14 d, ~2 × 10⁵ cells (100 μl volume) were stained with pooled peptide PE-conjugated tetramers for 60 min at 37°C. Subsequently, cells were stained with CD4-PerCP (BD Biosciences, Mountain View CA), CD3-FITC, and CD25-allophycocyanin mAbs (eBioscience, San Diego, CA) and analyzed by flow cytometry. Cells that gave positive staining were analyzed again using the corresponding individual peptide tetramers.

Tetramer assay for PDC-E2 peptides

To investigate T cell responses to putative PDC-E2 epitopes, CD4+ T cells were isolated from the PBMCs of vaccinated healthy DR0801 subjects by negative selection using a magnetic isolation kit (Miltenyi Biotec). Adherent cells from the CD4+ fraction were used as APCs. CD25+ T cells were depleted, as previously described, to encourage the outgrowth of autoreactive T cells (18). Two million CD4+CD25+ T cells/well were stimulated with individual PDC-E2 peptides that had been shown to bind to DR0801. After 14 d, ~2 × 10⁵ cells were stained (100 μl volume) with PE-conjugated tetramers and analyzed as described in the previous section.

Ex vivo frequency analysis of CD4+ T cells

Ex vivo analysis of CD4+ T cells was carried out as described previously (19). Briefly, 30 million PBMCs in 200 μl T cell culture medium were stained with 20 μg/ml PE-labeled tetramers at room temperature for 100 min. Cells were then stained with anti-CD4-allophycocyanin (eBio-science), anti-CD14 PerCP (BD Pharmingen), anti-CD19 PerCP (BD Pharmingen), and anti-CD45RA FITC for 20 min at 4°C. Cells were washed and incubated with anti-PE magnetic beads (Miltenyi Biotec) at 4°C for 20 min, washed again, and a 1/100th fraction was saved for analysis. The other fraction was passed through a magnetic column (Miltenyi Biotec). Bound, PE-labeled cells were flushed and collected. Cells in the bound and precolumn fractions were stained with Via-Probe (BD Bioscience) for 10 min before flow cytometry. Data were analyzed by gating on forward scatter/side scatter and excluding CD14+ and CD19+, and Via-Probe* (dead) cells and frequencies were calculated based on the number of tetramer-positive cells and the calculated number of CD4+ T cells.

T cell clone isolation and assessment of proliferation

T cell lines were generated by staining T cells with tetramer directly ex vivo and then sorting gated tetramer-positive CD4+ cells using a FACSaria (at single-cell purity) and expanding in a 96-well plate in the presence of 1.0 × 10⁶ irradiated PBMCs and 2 μg/ml PHA (Remel, Lenexa, KS). T cells were stained with tetramers that had been loaded with various substituted versions of the H1MP98–110 peptide, as well as stimulated in parallel with each of these peptides (10 μg/ml), adding HLA-DR–matched irradiated PBMCs as APCs. After 48 h, each well was pulsed for an additional 16 h with 1 μCi [3H]thymidine (Amersham Biosciences, Piscataway, NJ), and uptake was measured with a scintillation counter to assess proliferation.

Peptide-binding competition

Various concentrations of each test peptide were incubated in competition with 0.1 μM biotinylated H1MP98–110 peptide in wells coated with DR0801 protein or in competition with 0.1 μM biotinylated H1HA96–118 peptide in wells coated with DR1101 protein, as previously described (20). After washing, the residual biotin-labeled peptide was detected using europium-conjugated streptavidin (PerkinElmer, Waltham, MA) and quantified using a Victor2D time-resolved fluorometer (PerkinElmer). Peptide-binding curves were simulated by nonlinear regression with Prism software (Version 4.03; GraphPad Software, La Jolla, CA) using a sigmoidal dose-response curve. IC50 binding values were calculated from the resulting curves as the peptide concentration needed for 50% inhibition of reference peptide binding. Experimentally determined relative binding affinity (RBA) values were taken as the IC50 value of the unsubstituted peptide divided by the IC50 value of the substituted peptide. The ratio of two IC50 values reflects the fold difference in peptide-binding affinity.

Molecular modeling

Models of DR0801 in complex with the H1MP98–110 sequence and selected variants of interest were prepared on a Silicon Graphics Fuel work station using the program Insight II, version 2005 (Accelrys, San Diego CA), essentially as described previously (21). Energy minimization was performed at pH 5.4, the experimental pH used for binding studies. The crystal structure of HLA-DRB1*0401 in complex with the collagen II peptide (22) at pH 5.4, the experimental pH used for binding studies. The crystal structure of HLA-DRB1*0401 in complex with the collagen II peptide (22) was used as the base molecule for all simulation studies. Figures were drawn with the aid of WebLab Viewer version 3.5 and DSViwer version 6.0 (Accelrys), using previously published formatting and color conventions (23).

Epitope prediction

To predict the binding affinity to DR0801 for peptides of interest, we used an approach similar to our published work (23). The calculated RBA (RBAc) of any peptide was calculated as the product of the observed RBA value for the substituted H1MP98–110 peptide with the equivalent residue in that position within that pocket (for the best possible binding register) or an interpolated value (for amino acids not measured) estimated based on
observed values for chemically similar amino acids. This can be expressed using the following formula:

$$\text{RBAc} = \frac{\text{RBA}_{p1} \times \text{RBA}_{p4} \times \text{RBA}_{p6} \times \text{RBA}_{p9}}{\text{RBA}_{p1}}$$

To predict minimal epitopes within DR0801-restricted epitopes, the RBAc was compared for all possible registers within the peptide. To predict minimal epitopes within DR1101-restricted epitopes, the published ProPred algorithm was used to score the possible registers within each peptide sequence (24).

**Cooperativity calculations**

Cooperative effects ($\Delta K_b$) were calculated as described by Ferrante and Gorski (25), taking the ratio of the expected effect of the two single substitutions (RBA) to the experimentally measured effect of the double substitution (RBAc). Cooperativity values < 1 indicate enhanced binding compared with the expected effect of the single substitutions.

**Results**

**Tetramer-guided epitope mapping of DR0801- and DR1101-restricted epitopes**

DR0801-restricted epitopes within H1HA, H3HA, H1MP, H1NP, TTL, and TT proteins were identified using the tetramer-guided epitope-mapping approach. Representative results for these epitope-identification experiments are shown in Fig. 1. For H1MP, positive staining was observed for peptide pool 3 (Fig. 1A) and for one individual peptide within that pool (Fig. 1B), identifying H1MP$_{89-108}$ as a peptide that contains a DR0801-restricted epitope. The sequences of these peptides are summarized in Table I. Using the same approach, DR1101-restricted epitopes were identified within H1HA, H1MP, H1NP, and TTL proteins (representative results are shown in Supplemental Fig. 1). We had previously identified DR1101-restricted epitopes within TT (26). The sequences of these peptides are summarized in Table II. Comparing the DR0801-restricted epitopes (Table I) and DR1101-restricted epitopes (Table II) within these viral Ags, DR0801- and DR1101-restricted epitopes were present within seven “shared” peptides. These “shared” peptides were present within the H1HA, H1MP, TTL, and TT peptides but not the H1NP peptide set. These results indicate that 7 of 19 DR0801-restricted epitopes (~37%) can be presented by DR1101 and, analogously, that 7 of 31 DR1101-restricted epitopes (~23%) can be presented by DR0801.

**Binding register of the study peptide**

Among the peptides identified as DR0801-restricted epitopes, H1MP$_{89-108}$ was selected as a convenient peptide to determine the binding preferences of DR0801, primarily because it appeared to contain a single binding register. Early results with truncated peptides suggested that the minimal binding residues occurred at the C-terminal end of the H1MP$_{89-108}$ peptide; therefore, the H1MP$_{98-110}$ peptide (a region in which the minimal binding region is centered within the peptide) was used to design a set of substituted peptides, with arginine at most positions but replacing positive charges in that sequence with both Y and E. As shown in Fig. 2, multiple non-conservative substitutions, including 100R, 103R, 105Y, and 105E, markedly reversed the binding of the peptide, suggesting that these represent anchor positions. The spacing of these residues establishes 100Y as the pocket 1 anchor, 103L as the pocket 4 anchor, 105R as the pocket 6 anchor, and 108T as the pocket 9 anchor.

**Determining binding preferences for DR0801**

To determine the preferences for the binding pockets of DR0801, a panel of H1MP$_{98-110}$-derived peptides with single amino acid substitutions was synthesized, including obligatory anchor residues for pocket 1 and general classes of amino acids for pockets 4, 6, and 9. Each of these peptides was bound to recombinant DR0801 protein in competition with the biotinylated reference peptide. The sequences of these peptides and their experimentally measured relative binding affinities (RBA) are summarized in Fig. 3 and Supplemental Table I. Pocket 1 accommodated Phe, Trp, and Tyr, followed by Leu, Ile, and Met, while tolerating Val. Pocket 4 had narrow preferences, preferring Met, Leu, Ala, and Pro but tolerating Arg, Val, and Tyr. Pocket 5 had somewhat broader preferences, accepting Ser, Arg, and Lys and tolerating Asp, His, Leu, Glu, Pro, Gln, Met, Val, and Tyr but excluding Thr and Asn. Pocket 9 had a strong preference for acidic residues (Asp and Glu) and accommodated Ala, Thr, Val, His, Pro, Gln, Met, Leu, Arg, and Tyr, while tolerating Asn and barely accepting Lys. As shown in Supplemental Fig. 2, disfavored amino acid substitutions at proposed anchor positions led to parallel decreases in tetramer staining and T cell proliferation, further corroborating our conclusions about the DRB1*0801 motif.

**Modeling analysis of the DR0801–peptide complex**

To visualize its binding pockets, models of the H1MP$_{98-110}$ peptide (KLYKLKREITFH) in complex with DR0801 were prepared as described in Materials and Methods (Molecular modeling). Pocket 1 was not modeled, because the residues that comprise this pocket are identical to DR0401, which is the basis for our model. As shown in Fig. 4A, pocket 4 is a restrictive pocket, because of its unusual combination of $\beta$-chain residues (Gly$_{13}$, Phe$_{26}$, Asp$_{28}$, Arg$_{32}$, Leu$_{50}$, Tyr$_{58}$) found only in DRB1*0801 alleles. Residues form pairs according to their hydrophobicity/hydrophilicity (Phe$_{26}$ with Leu$_{50}$ and Asp$_{28}$ with Arg$_{32}$), with the first pair pointing into the pocket and the second hydrophilic pair pointing into the

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**FIGURE 1.** Tetramer-guided epitope mapping of influenza matrix protein epitopes. (A) Pool mapping: T cells from a DR0801 donor were stimulated with overlapping peptide mixtures spanning H1MP and stained using pooled peptide–loaded tetramers after 2 wk. Peptide pool 3 gave positive staining. (B) Individual peptide mapping: T cells from the positive panel shown in (A) were stained again using tetramers loaded with each individual peptide from peptide pool 3. Peptide H1MP$_{89-108}$ was identified as a peptide that contains a DR0801-restricted epitope.
solvent. Binding of mutated peptides shows preference in this pocket for Leu, Met, Pro, and Val, consistent with the observed orientation of the residues in the pocket.

Pocket 6 contains many acidic residues (including $\beta$9Glu, which can turn toward this pocket when occupied by a basic residue, particularly if the $\beta$9 residue is not basic) and, thus, easily accommodates Arg (Fig. 4B). Interestingly, this pocket also accommodates Asp well and Glu to a lesser extent (Supplemental Fig. 3A). In this case, $\beta$9Glu turns away from pocket 6 and remains in pocket 9 because pocket 6 is occupied by an acidic residue. It is probable that water molecules act as hydrogen bond bridges between anchor and pocket residues, as was documented.

### Table I. Motif analysis for novel DR0801 epitopes

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>RBAc</th>
<th>Charges$^a$</th>
</tr>
</thead>
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<tr>
<td>H1HA$^a$ 126-142$^b$</td>
<td>SSFERFEFPKESSWPN</td>
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<td>2</td>
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<td>H1HA$^a$ 238-254</td>
<td>DQERGRNYYWNLLEFGD</td>
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<td>H1HA$^a$ 316-332$^b$</td>
<td>IGECPYVRSAKLRYMVT</td>
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<td>H1HA$^a$ 322-338$^b$</td>
<td>YVRSAKLRYMTGGLNIP</td>
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<tr>
<td>H1HA$^a$ 334-350</td>
<td>LEMPSIQSRGFLFGAAL</td>
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<td>H1HA$^a$ 340-356</td>
<td>IFQSLFGAGIATGEFEG</td>
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<td>VIEMKNTQFXTAVGKEFN</td>
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<td>TQFTAVGKEFNKLERRRM</td>
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<td>GKEFKNKLERRMNLK</td>
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<td>YVRSAKLRFMVTGLRNIP</td>
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<td>H1MP$^b$ 89-108</td>
<td>DPNNMDKA VKL</td>
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<td>H1MP$^a$ 97-116</td>
<td>VKL YRKLKREIT</td>
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<tr>
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<td>LNYRYYNLKFKIKRYT</td>
<td>0.73</td>
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</table>

Primary binding registers are indicated in bold type. Secondary registers or weak primary binding registers are underlined. 

$^a$Number of charged anchor residues in predicted register. 

$^b$Shared between DR0801 and DR1101.

### Table II. Motif analysis for DR1101 epitopes

<table>
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<th>Peptide</th>
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<th>Score</th>
<th>Charges$^a$</th>
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<td>QLSYVSSFERFEFPKESSW</td>
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<td>ERFIEFPKESSWPNHTVTGV</td>
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<td>YRNLLWLTGNGNLPLSNKS</td>
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<td>H1HA$^a$ 313-332$^b$</td>
<td>PVTIEGECPVYRASKLRYMV</td>
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</tr>
<tr>
<td>H1HA$^a$ 319-338$^b$</td>
<td>CPKVYRASKLRYMTGLRNIH</td>
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<td>1</td>
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<td>VLMEWLKTRPSTLPTKIG</td>
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<td>VLYRKLKREITFVGHKAEIS</td>
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<td>TNPLIRHENVMLASTTAK</td>
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<td>LIPVASSKSDQVKNITIDYM</td>
<td>0.03</td>
<td>1</td>
</tr>
<tr>
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<td>1.5</td>
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<td>TTL$^{12}$ 1242-1261$^b$</td>
<td>AVLKEEKLTSVLQKLYDDK</td>
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<td>2</td>
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<td>TSVQQLKLYDDKNNASLGLVG</td>
<td>0.9</td>
<td>2</td>
</tr>
</tbody>
</table>

Primary binding registers are indicated in bold type. Secondary registers or weak primary binding registers are underlined.

$^a$Number of charged anchor residues in predicted register.

$^b$Shared between DR0801 and DR1101.
in other HLA II–peptide complexes for this pocket (27). Pocket 9 has a wide set of preferences because it consists of residues that do not restrict its preferences. As shown in Fig. 4C, the polar Thr residue is well accommodated, surrounded by diverse residues. The presence of β57Ser also allows acidic, basic, aliphatic, and aromatic residues, as well as proline to anchor (for examples see Supplemental Fig. 3B, 3C). The presence of β57Asp would fundamentally alter the preferences of this pocket. The absence of a salt bridge between β57Asp and α76Arg strongly favors the binding of acidic residues, because the positive charge of the free α76Arg residue can interact with the anchored residue. In total, the modeling results demonstrate a molecular basis for the observed binding preferences for DR0801.

Motif analysis of peptides containing DR0801 epitopes

We identified a total of 19 peptides that contain DR0801-restricted epitopes (Table I). To predict minimal epitopes within these antigenic peptides, we used a prediction algorithm as described in Materials and Methods. For 17 of these antigenic peptides, the prediction algorithm identified a strong DR0801-binding motif. A few peptides contained two distinct registers that could be expected to bind DR0801. Two peptides (H1NP409–428 and TTL321–340) contained weak DR0801-binding motifs. For H1NP409–428, the key unfavorable residue in the best predicted motif is a Gln in pocket 4. For TTL321–340, the key unfavorable residue in the best predicted motif is a Gln in pocket 6. In general, these peptides contained a high proportion of charged and polar residues. More specifically, their minimal motifs contained as many as three charged residues within the predicted binding pockets.

We used the ProPred algorithm to predict minimal epitopes within the 31 peptides that contain DR1101-restricted epitopes (Table II). All but two of these antigenic peptides (H1MP98–110 and TT1130–1149) contained a strong DR1101-binding motif. A few peptides contained two distinct binding registers. The minimal motifs of these peptides contained as many as two charged residues within the predicted binding pockets. However, the DR0801-bound sequences contained a significantly higher number of charged anchors compared with DR1101-bound sequences (1.5/peptide versus 1.0/peptide, p = 0.03). This observed difference is consistent with the respective binding motifs of these two HLA class II proteins (summarized in Table III). Although these two motifs are similar for pockets 1 and 6, DR0801 is distinguished by its ability to accept Arg in pocket 4 and by its strong preference for acidic residues in pocket 9. As summarized in Table III, four of the six residues that differ between DR0801 and DR1101 are within binding pockets: two in pocket 4, one in pocket 7, and one in pocket 9, whereas the other two are in positions β16 and β58 (28).

Binding of peptides with dual substitutions to DR0801

In a previous study, we demonstrated (for DRB1*09:01 and DRB1*03:01) that the permissiveness of pocket 9 is dependent on pocket 6, apparently through the positioning of a charged β9 residue (29). The modeling results for DR0801 confirm the ability of β9Glu to deflect toward or away from pocket 9, depending on the charge of the residue anchored within pocket 6. To investigate possible interactions, we measured the binding characteristics for a panel of peptides with dual substitutions at the pocket 6 and pocket 9 positions, calculating cooperative effects (ΔKD) using the method of Ferrante and Gorski (25), as described in Materials and Methods. These results are summarized in Table IV. When a positively charged Arg residue is anchored at position 6, β9Glu moves away from pocket 9, creating a pocket that prefers acidic residues. This effect is less pronounced when a smaller, less basic Lys residue is present at position 6 (ΔKD = 1.7). A negatively charged Glu residue at position 6 pushes β9Glu toward pocket 9, reducing its ability to accommodate acidic residues (ΔKD = 7.6) and large hydrophobic residues (ΔKD = 8.0) while increasing its accommodation of basic residues (ΔKD = 0.67). Interestingly, a small uncharged Ser residue at position 6 creates a pocket 9 that accommodates basic residues (ΔKD = 0.13) but dramatically reduces its accommodation of acidic (ΔKD = 18) and large hydrophobic residues (ΔKD = 14). Based on these findings, it might be expected that peptides with a neutral pocket 6 residue, such as TTL321–340, could bind better than predicted by our standard algorithm.

T cell responses to PDC-E2 peptides

Given the strong association between DR0801 and PBC and the importance of the E2 subunit of PDC in disease, we next identified sequences from PDC-E2 that contain possible DR0801-binding motifs and measured the binding of these sequences to DR0801 and DR1101 (peptide sequences and measured binding affinities are shown in Table V). Although four of these peptides bound to DR0801, and five peptides bound to DR1101, only a single peptide bound to both proteins. A previous study from our group demonstrated that autoreactive T cells (specific for multiple self-Ags) are present in healthy individuals and that responses to these
epitopes can be readily detected when CD25+ T cells (putative regulatory T cells) are removed prior to in vitro culture (18). To investigate the antigenicity of potentially self-reactive epitopes, CD4+ T cells from healthy subjects with DR0801 haplotypes and DR1101 haplotypes were depleted of CD25+ cells and stimulated with PDC-E2 peptides. After 2 wk in culture, these cells were analyzed using the corresponding DR0801 or DR1101 tetramers. Staining results for a representative DR0801 subject are shown in Fig. 5A. In this particular subject, two peptide sequences (PDC-E2249–263 and PDC-E2629–643) were positive. Results for a representative DR1101 subject are shown in Fig. 5B. A summary of the staining results for all of the DR0801 subjects is shown in Fig. 5C. In total, positive responses were observed in multiple DR0801 subjects for three of the four PDC-E2 peptides, implicating these as putative autoreactive T cell epitopes. As expected, responses to these peptides were not seen in DR1101 subjects. We performed direct ex vivo tetramer staining to further corroborate the specificity of our tetramer staining. Representative results for one DR0801 subject are shown in Fig. 5D. Tetramer-labeled cells were virtually undetectable in the unenriched sample but were revealed as distinct populations following enrichment. As expected, H1MP98–110-specific T cells were frequent and CD45RA+ (suggesting a memory phenotype), whereas PDC-E2–specific T cells were infrequent and CD45RA- (suggesting a naive phenotype). Similar results were obtained for additional subjects (data not shown).

Discussion
The amino acid sequences of HLA class II proteins dictate the binding specificity for each of their binding pockets. The specificity of these pockets, in turn, dictates the peptide sequences that are presented to CD4+ T cells, both in the thymus and the periphery, thereby shaping the CD4+ T cell repertoire. DR0801 and DR1101 have similar sequences and yet DR0801 is associated with PBC (4, 5) whereas DR1101 is protective. We hypothesized that key polymorphic residues, such as β57Ser, confer distinct peptide-binding preferences for DR0801, favoring the presentation of unique peptides (that are not presented by the homologous protective DR1101 protein) from self-Ags, such as PDC-E2, and selecting a potentially autoreactive T cell repertoire. A detailed characterization of the binding specificity for the binding pockets of DR0801 was necessary to address this hypothesis. Our empirical observations and modeling results suggested an arrangement of pocket residues that favor the binding of aliphatic and, to a lesser extent, Arg residues in pocket 4 (and also a unique accommodation of proline) and of both basic and acidic residues in pocket 6. The preferences of pocket 9 were profoundly influenced by the presence of a non-Asp residue at β57, in contrast to DR11, which is β57Asp

Most HLA class II molecules are stabilized by the formation of a salt bridge between β57Asp and α76Arg, residues that (along with β9, β30, β37, α69, α72, and α73) dictate the size and preferences of binding pocket 9. It was shown that nearly all HLA-DR and HLA-DQ molecules that lack β57Asp favor the binding of acidic residues, because the positive charge of the free α76Arg residue can interact with the anchored residue (11) (http://www.syfpeithi.de, accessed on 16 January 2012). We observed that DR0801 strongly favored acidic residues (Asp and Glu) in pocket 9 when a basic residue is anchored in pocket 6. However, when a polar or acidic residue was anchored in pocket 6, this preference was reduced. Similar preferences were recently reported by Muixi et al. (30), who inferred a binding motif for DR8 based on an
aligned matrix of 228 unique sequences that were eluted from HLA-DR sequences purified from a HLA-DR8 homozygous lymphoblastoid cell line. Their findings indicated a preference for basic and small aliphatic residues in pocket 4, basic residues in pocket 6, and acidic residues in pocket 9. These preferences were distinct from those of DR1101. Although these highly homologous alleles share identical sequences and preferences for pocket 1 (dictated almost exclusively by the β86 residue), their sequences differ at important pocket residues, including β13 (Gly versus Ser), β74 (Leu versus Ala), and β57 (Ser versus Asp). Consequently, pocket 4 of DR0801 appears to be more accommodating toward Arg residues than the corresponding pocket of DR1101, and their preferences for pocket 9 are considerably different, with those for DR1101 being restricted to small aliphatic residues and proline (31) (http://www.syfpeithi.de, accessed on 16 January 2012). The subtle, but notable, differences in the binding preferences of DR0801 and DR1101 apparently have important immunologic consequences. A comparison of the DR0801- and DR1101-restricted T cell epitopes within influenza and tetanus Ags identified several “shared” epitopes, but not necessarily identical binding registers, with at least one present within each Ag tested (except for H1NP, which contained only distinct epitopes). In agreement with their binding motifs, DR0801-restricted epitopes contained more charged residues at anchor residues than did DR1101 epitopes. Interestingly, for DR1101, the distinct peptides had significantly fewer charged anchor residues than did the shared peptides (0.8/peptide versus 1.7/peptide, p = 0.006), whereas for DR0801, the “shared” and distinct epitopes had equivalent numbers of charged anchor residues (1.2/peptide versus 1.7/peptide, p = 0.24).
Although these two HLA-DR proteins presented several of the same viral epitopes, DR0801 was able to bind and present unique PDC-E2 peptides. We identified four PDC-E2 peptides that bound to DR0801 and five PDC-E2 peptides that bound to DR1101. However, only a single peptide bound to both proteins, a result that is consistent with the predicted binding of these sequences to DR1101. Three of the four PDC-E2 peptide sequences that were bound by DR0801 (PDC-E2145–159, PDC-E2249–263, and PDC-E2219–233) were able to elicit a high-avidity T cell response from multiple subjects with DR0801 haplotypes. However, none of these sequences elicited responses in subjects with DR1101 haplotypes. These results suggest selection of T cell specificities by DR0801 that are distinct from the highly homologous DR1101 motifs. Indeed, T cells specific for these peptides were present at low, but detectable, frequencies in the naive repertoires of healthy subjects with DR0801 haplotypes. Interestingly, these three immunogenic sequences each have a charged residue within one of the predicted binding pockets, whereas the nonimmunogenic PDC-E2269–283 peptide has no charges in its predicted pockets. One issue that may set apart DR0801 from DR1101 is the latter’s contribution of beta chain residue 57 in peptide binding ability of both HLA-DR and -DQ molecules.

Cumulatively, our findings suggest that DR0801 has a unique capacity to present charged self-peptides derived from PDC-E2 (and perhaps other hepatic Ags), leading to the selection of potentially autoreactive T cells that play a crucial role in the breakdown of self-tolerance in the liver. Thus, our study links well-documented genetic-susceptibility data with an immune mechanism of peptide presentation and T cell repertoire selection. In addition to its genetic-susceptibility data with an immune mechanism of presentation, the DR0801 complex with bound peptides, the β57-59DEE peptide, has a high density of charged amino acids, which may be expected to have higher stability compared with self-peptide/DR0801 complexes, thereby altering the selection of T cells that recognize self-Ags.

Consequently, given the high density of charged and polar amino acids set of peptides that is presented by DR0801 leads to the selection of regulatory T cells that suppress autoreactive T cell responses (10, 28). Cumulatively, our findings suggest that DR0801 has a unique capacity to present charged self-peptides derived from PDC-E2 (and perhaps other hepatic Ags), leading to the selection of potentially autoreactive T cells that play a crucial role in the breakdown of self-tolerance in the liver. Thus, our study links well-documented genetic-susceptibility data with an immune mechanism of peptide presentation and T cell repertoire selection. In addition to its binding preferences, DR0801 lacks the typical β57Asp; the consequent elimination of the salt bridge with α76Arg decreases the biochemical stability of the DR0801 heterodimer in comparison with other HLA-DR alleles. This fundamental difference in biochemical stability may alter the peptide-binding repertoire and consequently influence issues, such as tolerance to self-peptides (32). It may also be that, in addition to conferring stability to the complexes with bound peptides, the β57-59DEE sequence of the PBC-protective allele HLA-DRB1*1101 selects distinct TCRs that exclude key autoreactive specificities or that lead to the development of regulatory T cells that suppress autoreactive T cell responses (10, 28). Cumulatively, our findings suggest that the set of peptides that is presented by DR0801 leads to the selection of unique epitope repertoire recognizing peptide sequences that contain a high density of charged and polar amino acids. This, combined with its inherent lack of biochemical stability, gives rise to a reservoir of potentially autoreactive cells that may play a role in the breakdown of self-tolerance in PBC. As such, monitoring T cells that recognize these epitopes could provide a useful biomarker for evaluating the efficacy of therapeutic agents in subjects with PBC, particularly those that target immunologic pathways.

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Disclosures
The authors have no financial conflicts of interest.

References
FIGURE 5. T cell responses to PDC-E2 peptides. (A) Tetramer staining of cultured CD4+ T cells from a representative DR0801+ healthy subject. (B) Tetramer staining of cultured CD4+ T cells from a representative DR1101+ healthy subject. (C) Cumulative tetramer staining results for PDC-E2 responses in multiple healthy DR0801+ subjects. Tetramer-positive populations >0.4% above background were considered positive (indicated by dotted line). (D) Direct ex vivo tetramer analysis of CD4+ T cells from a representative DR0801+ healthy subject. Staining of nonenriched PBMCs, for which staining is undetectable (upper left panel). Remaining panels depict staining of anti-PE–enriched PBMCs for H1MP89–108–specific, PDC-E2 145–159–specific, and PDC-E2 256–269–specific CD4+ T cells versus CD45RA.


