Published in final edited form as:

Mol Immunol. 2012 February ; 50(1-2): 26–34. doi:10.1016/j.molimm.2011.11.014.

DRB1*12:01 presents a unique subset of epitopes by preferring aromatics in pocket 9

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Keywords

HLA-DR12; peptide motif; Class II MHC; structural modeling; T cells; Antigens/Peptides/Epitopes

1. Introduction

CD4⁺ T cells recognize immunogenic peptides that are bound and presented by HLA class II molecules on the surface of antigen presenting cells. Because the peptide binding pockets of class II molecules include polymorphic residues, each class II allele binds and presents a distinct set of peptides as determined by the distinct pockets within its peptide binding cleft. Recent efforts to predict peptide binding to diverse HLA class II alleles with minimal input data have been reasonably successful (Nielsen et al. 2010). However, for class II alleles that are prevalent and of immunologic interest, binding motif studies can provide important information, allowing a more detailed understanding of the peptides presented and the precise binding register of the corresponding epitopes. DRB1*12:01 (DR1201) is a common allele in Asian populations (Gonzalez-Galarza et al. 2011). It is also of immunologic interest, based on its study in the context of susceptibility to (Cerna et al. 1994; Liao et al. 2007; Albert and Scholz 1998) or protection from (Kapitány et al. 2005) various forms of inflammatory arthritis, susceptibility to sarcoidosis (Grunewald 2010), and its ability to present tumor antigen CAMEL (Slager et al. 2003) and hepatitis C antigen NS3 (Diepolder et al. 1997). Historically, DR12 alleles have been classified together with DR11, as both belong to the older HLA-DR5 serotype group and share a high degree of amino acid sequence similarity (97.8% identity). However, unlike most HLA class II molecules

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(including DR11) DR1201 does not have the classical aspartic acid residue at the β 57 position. Therefore, DR1201 cannot form a salt bridge at its pocket 9. Lack of this inter-chain salt bridge has been shown to significantly influence the anchor residue selection at pocket 9, the stability of the MHC II-peptide complex, and in some cases TCR interaction (Kwok et al. 2008; Hovhannisyanyan et al. 2008). Therefore, we hypothesized that this feature would cause DR1201 to exhibit a peptide binding motif that is distinct from that of the closely-related DRB1*11:04 (DR1104) allele. Correspondingly, the objective of this study was to identify the complete peptide binding motif of DR1201 using an in vitro peptide competition assay complimented by a structural modeling approach. The resulting peptide binding preferences of DR1201 were used to identify minimal T cell epitopes within antigenic peptides that were identified by tetramer guided epitope mapping, and compared to the reported motif for DR1104 (Newcomb and Cresswell 1993; Verreck et al. 1996).

2. Methods

2.1. Peptides

Panels of overlapping peptides derived from Influenza A/New Caledonia/2-/99 Hemagglutinin (H1HA), Influenza A/Puerto Rico/8/34 Matrix Protein M1 (H1MP), Influenza A/Puerto Rico/8/34 Nucleoprotein (H1NP), Tetanus Toxin Light Chain (TTL) or Tetanus Toxin Heavy Chain (TT) were synthesized by Mimotopes (Clayton Australia). The biotinylated reference matrix protein peptide MP₄₀₋₅₄ (EVLMEWLKTRPILSP) was synthesized by Genscript with one 6-aminohexanoic acid spacer added between the N-terminal biotin label and the remainder of the peptide sequence. A panel 15-mer peptides with sequences based on the MP₄₀₋₅₃ sequence was synthesized by Sigma (PEPScreen, Sigma-Aldrich). Each peptide was dissolved in DMSO at 20 mg/ml and subsequently diluted as needed.

2.2. DR1201 protein and tetramers

Recombinant DR1201 protein was produced as previously described (Novak et al. 1999). Briefly, soluble DR1201 was purified from insect cell culture supernatants by affinity chromatography and dialyzed against phosphate storage buffer, pH 6.0. For the preparation of HLA class II tetramers, DR1201 protein was biotinylated at a sequence-specific site using biotin ligase (Avidity, Denver, CO) prior to dialysis into phosphate storage buffer. The biotinylated monomer was loaded with 0.2 mg/ml of peptide by incubating at 37°C for 72 hours in the presence of 2.5 mg/ml n-octyl- β -D-glucopyranoside and 1 mM Pefabloc SC (Sigma-Aldrich, St. Louis, MO). Peptide loaded monomers were subsequently conjugated as tetramers using R-PE streptavidin (Biosource International, Camarillo, CA) at a molar ratio of 8 to 1.

2.3. Human Subjects

Healthy DR1201 subjects were recruited with written consent as part of an IRB approved study.

2.4. Tetramer-guided epitope mapping

The tetramer-guided epitope mapping procedure was conducted for each protein as previously described (Novak et al. 2001). PBMC were isolated from the blood of vaccinated healthy DR1201 subjects by Ficoll underlay. Subsequently, CD4⁺ T cells were isolated using the Miltenyi CD4⁺ T cell isolation kit. Cells recovered from the CD4⁻ fraction were incubated in 48 well plates (3×10^6 cells per well) for 1 h and washed, leaving adherent cells as APC. After adding 2.5 million CD4⁺ T cells per well, each well was stimulated with a pool of five consecutive peptides spanning the H1HA, H1MP, H1NP, TTL and TT protein

sequences. After 14 days, 100 μ l of resuspended cells were stained with pooled peptide PE-conjugated tetramers for 60 min at 37 °C. Subsequently, cells were stained with CD4-PerCP (BD Biosciences), CD3-FITC and CD25-APC mAbs (eBioscience) and analyzed by flow cytometry. Cells from pools that gave positive staining were analyzed again with the corresponding individual peptide tetramers to identify each antigenic peptide.

2.5. Peptide binding competition

Various concentrations of each test peptide were incubated in competition with 0.04 μ M biotinylated MP₄₀₋₅₄ peptide in wells coated with containing HLA-DR1201 protein as previously described (Ettinger and Kwok 1998). After washing, the biotin-MP peptide was labeled using europium-conjugated streptavidin (Perkin Elmer) and quantified using a Victor2 D time resolved fluorometer (Perkin Elmer). Peptide binding curves were simulated by non-linear regression with Prism software (Version 4.03, GraphPad Software Inc.) 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. Relative binding affinity (RBA) values were calculated 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.

2.6. Molecular modeling

Models of DR1201 with the H1MP₄₀₋₅₃ 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, USA), essentially as previously described (Reichstetter et al. 2002). Energy minimization at 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 (Dessen et al. 1997) was used as the base molecule for all simulation studies. Figures were drawn with the aid of WebLabViewer version 3.5 and DSViewer Pro version 6.0, of Accelrys using previously published formatting and color conventions (James et al. 2008). The pdb coordinates are freely available to interested researchers upon requests to WWK or GKP.

2.7. Epitope Prediction

To predict minimal epitopes within antigenic peptides we utilized the approach from our published work for DRB1*1001 (James et al. 2010). An array of binding coefficients (C_p) was developed for each pocket based on the observed binding of single (at pocket 1, 4, 6, or 9) amino acid substituted peptide sequences. These C_p values are summarized in Supplementary Table I. Because all possible amino acids were not measured for any of the pockets, missing values in the data set were estimated based on the observed values for chemically similar amino acids. Peptide binding affinities were calculated based on the following formula:

$$RBA = C_{p1} \times C_{p4} \times C_{p6} \times C_{p9}$$

In this formula, each C_p refers to the observed or estimated binding coefficient. To predict RBA values for peptides, C_p values were taken from the table using lookup procedure by scanning across every possible binding register and taking the highest observed RBA value for the sequence.

3. Results

3.1 Tetramer-guided epitope mapping of DR1201 restricted epitopes

DR1201-restricted epitopes within Influenza A/New Caledonia/2-/99 Hemagglutinin (H1HA), Influenza A/Puerto Rico/8/34 Matrix Protein M1 (H1MP), Influenza A/Puerto Rico/8/34 Nucleoprotein (H1NP), Tetanus Light Chain (TTL) and Tetanus Toxin Heavy Chain (TT) proteins were identified using tetramer-guided epitope mapping approach. CD4⁺ T cells from DR1201 subjects were stimulated using pools of overlapping peptides spanning each protein of interest and stained with the corresponding pooled peptide tetramers after two weeks in culture. Pools that were tetramer positive were stained again using individual peptide tetramers to reveal the antigenic peptides within each pool. Representative results for these epitope identification experiments are shown in Fig. 1. For H1MP, positive staining was observed for peptide pools 1 and 2 (Fig. 1A) and for one individual peptide within each of these pools (Fig. 1B), identifying H1MP₃₃₋₅₂ and H1MP₄₁₋₆₀ as peptides that contain DR1201 restricted epitopes. Using this approach, a total of 15 peptides were identified from selected foreign antigen proteins (upper portion of Table I). Among these peptides, the overlap region of H1MP₃₃₋₅₂ and H1MP₄₁₋₆₀, namely residues 40-54, was chosen as a study peptide to determine the binding preferences of DR1201.

3.2 Binding Register of the study peptide

The binding register of the study peptide, MP₄₀₋₅₄, was confirmed using a set of arginine-, tyrosine-, or glutamic acid-substituted peptides. Non-conservative replacement of anchor amino acid residues has been previously shown to disrupt peptide binding (Ettinger et al. 2006). As shown in Figure 2, arginine substitutions at residues 43, 46, 48, and 51 prevented peptide binding, implying that these represent anchor positions for binding to DR1201. The spacing of these residues implicates 43M as the P1 anchor, 46L as the P4 anchor, 48T as the P6 anchor, and 51I as the P9 anchor.

3.3 Determining Binding Preferences of DR1201

To determine the amino acid preferences for the binding pockets of DR1201, a panel of MP₄₀₋₅₃ derived peptides with single amino acid substitutions was synthesized. These substitutions represented obligatory anchor residues for pocket 1 and general classes of amino acids for pockets 4, 6, and 9. Each of these peptides was bound (at various concentrations) to recombinant DR1201 protein in competition with the biotinylated MP₄₀₋₅₄ reference peptide. The sequences of these peptides and their relative binding affinities (RBA) are summarized in Table II. Corresponding to these observed binding affinities, the preferences for each pocket are summarized in Figure 3. Pocket 1 accommodated Ile, followed by Leu, Met, Phe, and Val. Given that Tyr was unable to bind, Trp would presumably also be too bulky. Pocket 4 had narrow preferences, preferring Leu, Ile and Met but tolerating Lys and barely tolerating Arg. Pocket 6 had extremely broad preferences tolerating Arg and excluding Glu, while accepting a wide range of other residues. Pocket 9 mainly preferred bulky, polar, and acidic residues, preferring Tyr, Phe, Met, Ile, Gln, and Glu, but also accommodated Val and tolerated Asp, Ser, and Asn. For this analysis, values within 2.5-fold of the reference peptide (RBA = 0.4) were preferred, values within 20-fold of the reference peptide (RBA = 0.05) were tolerated, and values less than 20-fold of the reference peptide (RBA = 0.05) were excluded.

3.4 Modeling analysis of peptides in complex with DR1201

To visualize the binding pockets of DR1201, models of the H1MP₄₀₋₅₃ peptide (EVLMEWLKTRPILS) in complex with DR1201 were prepared as described in Materials and Methods. Modeling results for the unmodified peptide (Figure 4A) indicated that 43M is

in pocket 1, 48T in pocket 6, and 51I in pocket 9 in an orientation that suggests high affinity binding. These results verify the binding register of the H1MP₄₀₋₅₃ peptide and its substituted derivatives. Bound in this register, 45W fits into the p3 shelf as first shown by Wiley for p3Met of the CLIP/DR3 complex (Ghosh et al. 1995). Figure 4B depicts pockets 4 and 6 of the complex. The unique set of residues in pockets 4 and 6, β 28Asp/ β 30His/ β 70Asp/ β 71Arg induces an arrangement where β 71Arg orients itself towards β 28Asp, and β 30His is likewise attracted to β 28Asp, remaining positively charged at endosomal pH (pKa of histidine = 6.0-6.5), as well as at extracellular pH (upon fusion onto the cell membrane). As the bottom of these two pockets is composed of two of the smallest residues, b13Gly for p4 and b11Ser for p6, the hydrophobic anchor at p4 will also constitute a “wall” for pocket 6. Pocket 6 is essentially composed of several hydrophilic residues, of which β 30His and β 71Arg are attracted to β 28Asp, and by contrast α 11Glu and α 66Asp repel each other (Figure 4B); therefore it is not surprising that this pocket can accommodate to varying degrees a variety of aromatic, aliphatic, charged and polar residues. The ample room left in pocket 6 by β 13Gly allows for the anchoring of bulky residues such as Tyr and perhaps even Trp. The close proximity between the residue anchored in pocket 6 and the residue anchored in pocket 4 that is indicated by the model suggests that similar residues in these respective pockets could stabilize one another. The depicted arrangement, in which the residue in pocket 6 approaches as close as possible to the p4Leu residue, favors a hydrophobic residue. This explains the observed preference for Ala, Met, and Phe. However, the anchoring of a basic residue in pocket 4 (Arg and Lys are both tolerated) could influence the preferences of pocket 6, increasing its accommodation of basic and polar residues. In addition, it is expected that the hydrophilic pocket residues would be solvated and thus better stabilized than the model is able to indicate. Pocket 9 accepts polar and acidic residues, as expected for alleles that lack Asp at β 57, but (as depicted in Figure 4C) the combination of hydrophobic and mostly flexible residues in this pocket (β 72Ile, β 73Met, β 9Glu, β 37Leu, β 57Val, β 61Trp) accommodate Tyr/Phe exquisitely. In total the modeling results demonstrate a molecular basis for the observed binding preferences for DR1201.

3.5 Motif Analysis of Peptides Containing DR1201 epitopes

As described in section 3.1 and summarized in the upper portion of Table I, we identified a total of 15 peptides that contain DR1201 restricted epitopes. To predict minimal epitopes within these antigenic peptides we utilized the algorithm described in section 2.7. For 14 of the antigenic peptides, single nonameric core epitopes consistent with the DR1201 binding motif were identified (boldface). A few peptides contained two distinct registers that could be expected to bind DR1201. For these the second motif is underlined. One peptide (H1NP109-125) was problematic because it contained only an extremely weak motif. To investigate the accuracy of our binding motif algorithm, we measured the binding affinity of these 15 peptides (Table I). Reassuringly, there was good agreement between the predicted binding affinities of these sequences and the observed binding. Of the four sequences with a predicted RBA of 0.1 or less, three of the four had a measured RBA less than 0.1. Similarly, six of the seven sequences with a predicted RBA greater than 1 had a measured RBA greater than 1. The four sequences with a predicted RBA between 0.1 and 1.0 all had a measured RBA within that range. These findings support the utility of our algorithm. The peptides with low binding affinities had unfavorable residues in at least one binding pocket. For example, H1HA 181-197 and H1HA 187-203 had an unfavorable His residue in pocket 9. H1NP 408-424 bound with somewhat higher affinity than expected. For this peptide's motif (**FSVQRNLPF**) the only problematic residue is the Gln in pocket 4. Given our modeling results, which indicate that the residue anchored in pocket 6 can be drawn toward the residue anchored in pocket 4, it is possible that the Asn residue in pocket 6 may stabilize the Gln residue in pocket 4. The same method was used to predict minimal epitopes within previously published DR1201 restricted epitopes. As summarized in the lower portion of

Table I, three of these epitopes contained strong motifs, one contained a weak motif, and two contained extremely weak motifs.

4 Discussion

The HLA class II binding cleft contains four pockets (pockets 1, 4, 6, and 9) which include polymorphic residues that define a peptide binding specificity for each HLA class II protein (Stern et al. 1994). This binding specificity, in turn, defines the set of peptides that are available for the thymic selection and peripheral activation of CD4⁺ T cells. As such, HLA class II molecules play a fundamental role in shaping CD4⁺ T cell-mediated immune responses. In particular, the binding specificity of DR1201 is of immunologic interest because of its moderate prevalence and documented association susceptibility to or protection from various forms of arthritis and sarcoidosis. However, the preferences for DR1201 had been largely uncharacterized and relatively few DR1201 restricted T cell epitopes are known (the IEDB currently lists only 5 positive T cell assays for DR1201) (Vita et al. 2010). In this work, we report 15 additional peptides that contain T cell epitopes restricted by DR1201 and a definitive binding specificity for its four major binding pockets. In the ensuing paragraphs, the preferences of each pocket will be considered in turn. This analysis relies heavily on molecular modeling to corroborate our empirical binding measurements. The detail and accuracy that protein simulation and molecular dynamics can achieve when applied appropriately (with adequate sequence homology and similarity in biochemical function) has been previously summarized (Grossfield 2011; Srinivasan and Blundell, 1993; Johnson et al. 1994). Along with the DR11 alleles, DR1201 is part of the HLA-DR5 serotype group, sharing considerable homology with DR1104 (Table III). As such, it can be expected that the binding preferences of DR1201 would be similar to DR1104 in some respects. Therefore, the similarities and differences between these alleles will be discussed. The observed motifs for these two alleles are summarized in Figure 5. DR1201 is also one of an appreciable minority of class II alleles that lack the classical β 57 Asp residue. Therefore, the contributions of the β 57 residue to the binding motif will also be discussed.

The binding properties observed for pocket 1 were as expected. The specificity for this pocket is dictated almost exclusively by the β 86 residue. All HLA-DR proteins contain either a Val or Gly residue at a β 86 and this dimorphism dictates the size of amino acids that can be accommodated in this pocket. DR1201 belongs to the first scenario; therefore, pocket 1 of DR1201 preferred aliphatic rather than bulky aromatic residues, although phenylalanine is accommodated well. DR1104 also has the same β 86Val residue, and prefers Ile, Leu, and Val at this position as DR1201 does; DR1101 has smaller Gly residue at β 86, and prefers bulky aromatic residues such as Tyr and Trp in its first binding pocket (Verreck et al. 1996). Unlike many HLA-DR alleles, DR1201 contains an Ala residue rather than the more common Val at β 85. This smaller residue may increase the size of pocket 1, explaining its excellent accommodation of Phe; on the other hand, the hydrophobicity of the pocket will not allow Tyr.

Similar to some other HLA-DR binding motifs such as DR1001, pocket 4 was the most restrictive pocket of DR1201 (James et al. 2010). This pocket preferred only Leu and Ile, also accepting Met, Lys, and barely Arg. The modeling results suggested a pocket that favored hydrophobic residues, as the walls of this pocket contain aliphatic amino acids and the two charged residues are able to move out of the pocket. For the anchoring of Lys and Arg, it would appear that the attraction of β 71Arg by β 28Glu, leaves β 70Asp alone at the rim of the pocket, and leads to a slightly acidic environment, allowing these basic residues to bind, albeit weakly. The modeling results also revealed a possible interaction between the residues anchored in pocket 4 and pocket 6 (via their close proximity), suggesting that

similar residues are more likely to be accommodated in these pockets. The residues that comprise pocket 4 of DR1201 and DR1104 are somewhat similar. DR1201 contains β 13Gly, β 26Leu, β 70Asp, β 71Arg, β 74Ala and β 78Tyr. DR1104 shares β 70Asp, β 71Arg, β 74Ala and β 78Tyr but contains β 13Ser, and β 26Phe. DR1104 has been shown to accept Leu, Val, Met, Ala, Phe, and Tyr at pocket 4 (Verreck et al. 1996). It is possible that β 26Phe better accommodates aromatic residues at this position, perhaps via aromatic-aromatic interactions.

Pocket 6 of DR1201 was the most permissive, accepting every amino acid residue except aspartate. Similar permissiveness was observed for pocket 6 of DR0901 (James et al. 2009). Pocket 6 is essentially composed of hydrophilic residues, easily explaining this pocket's ability to accommodate most charged and polar residues. However, the model indicates a large pocket 6 (in part because of β 13Gly) which favors the anchoring of bulky residues. In the model, the residue in pocket 6 approaches close enough to pocket 4 to be stabilized by the residue in that pocket, which would typically be an aliphatic residue. This and solvation of hydrophilic pocket residues by water promote the anchoring of hydrophobic residues. It was somewhat surprising that this pocket favors the binding of Glu while excluding Asp. In the pocket β 71Arg and β 30His are accessible to stabilize acidic residues while the presence of α 11Glu and β 28Glu could oppose their anchoring. Given their different lengths, this arrangement prohibits the anchoring of aspartic acid but allows glutamic acid to bind to pocket 6, because the charged group for the latter is able to reach farther within the pocket and perhaps form a hydrogen bond with β 11Ser. The residues that comprise pocket 6 of DR1201 and DR1104 are again quite similar, differing primarily at β 30 (His to Tyr respectively). This difference (tantamount to the removal of one partial positive charge, and allowing less space in the pocket) confers a far more acidic character to pocket 6 of DR1104, strongly favoring the anchoring of positively charged residues such as His, Lys and Arg at this pocket (Verreck et al. 1996). The acidic residues β 11Glu and β 66Asp can now form salt bridges with a positively charged anchor, diminishing in parallel their mutual repulsion.

Most HLA class II molecules are stabilized by the formation of a salt bridge between β 57Asp and β 76Arg, residues that profoundly influence the size and preferences of binding pocket 9. However, other residues, such as β 9, β 30, β 37, β 69, β 72 and β 73 also make important contributions to the preferences of this pocket. DR1201 has a valine rather than aspartic acid at β 57. HLA-DR and -DQ molecules that lack β 57Asp have been previously shown to favor the binding of acidic residues, as the positive charge of the free β 76Arg residue can interact with the anchored residue (Nepom et al. 1996). Alternatively, if the size and the physicochemical properties of the pocket permit, the absence of this salt bridge has been shown to favor the binding of aromatic residues (Ettinger et al. 2006; Quarsten et al. 1998; van de Wal et al. 1997). We observed that DR1201 strongly favored Tyr and Phe in pocket 9, also preferring aliphatics. As previously observed for HLA-DQ2 and DQ0604 (Ettinger et al. 2006; Quarsten et al. 1998; van de Wal 1997), DR1201 showed no particular preference for negatively charged residues, although acidic and polar residues were tolerated. The modeling results suggest that the absence of β 57Asp in combination with flexible residues in other pocket residues produces a large, open pocket that exquisitely accommodates aromatics. β 30His, while paired to β 28Asp, and the unpaired β 76Arg form a positively charged barrier for pocket 9, allowing the anchoring of polar and acidic residues while excluding basic residues. Pocket 9 did not accept short amino acids such as Ala and Pro, probably due to the large size of the pocket (although the rigidity of the Pro residue at position 8 of the reference peptide may constrain the conformations available for binding). The residues that comprise pocket 9 of DR1201 and DR1104 are fairly distinct, only sharing β 9Glu and β 61Trp while differing at β 30 (His and Tyr respectively), β 37 (Leu and Tyr respectively) and β 57 (Val and Asp respectively). Because of the intact salt bridge and the

larger Tyr residues, pocket 9 of DR1104 appears to be a smaller, more confined pocket, preferring small residues such as Ala, Gly, Ser and Pro (Verreck et al. 1996).

Among the 15 novel peptides that contain DR1201 restricted T cell epitopes, our study identified multiple pairs of peptides that appeared to contain the same core epitope. This provided an opportunity to examine the importance of flanking residues for optimal binding. In total four pairs of peptides were predicted to share the same core regions, with different terminal flanking sequences. For these pairs, H1MP₃₃₋₅₂/H1MP₄₁₋₆₀ and TT₁₂₃₄₋₁₂₅₃/TT₁₂₄₂₋₁₂₆₁ bound with nearly identical affinities. H1HA₁₈₇₋₂₀₃ bound with similar a slightly reduced affinity than H1HA₁₈₁₋₁₉₇. This subtle difference could probably be attributed to the consecutive proline residues (198 and 199) in the latter sequences. TTL₂₄₁₋₂₆₀ bound with lower affinity than TTL₂₄₉₋₂₆₈, indicating that C terminal flanking residues are needed for optimal binding in this instance. Therefore, the relative importance of flanking residues appears to vary for different peptide backbones.

Cumulatively, our findings define a peptide binding motif for DR1201 that is distinct from closely related HLA-DR5 alleles. It should be noted that one early study attempted to define a motif for DR1201 by aligning the sequences of a limited number of eluted peptides (Falk et al. 1994). This study correctly predicted a preference for aliphatic residues in pocket 1 and aromatic residues in pocket 9. However, no conclusions could be made concerning pocket 4. Based on our findings, this pocket appears to be the most restrictive pocket and therefore the most important for discriminating binders from non-binders. Given the observed binding preferences, it is likely that the set of peptides that is presented by DR1201 leads to the selection of a unique T cell repertoire. As reported in genetic association studies, the DR1201 haplotype protects against infection by Hepatitis virus (Meng et al. 2003; Romero et al. 2008). Therefore it would appear that the DR1201 restricted T cell repertoire is well suited to recognize this virus. While the motif we have described should accurately describe high affinity peptide ligands, self-peptides might be expected to deviate from this motif due to the constraints of thymic selection. For example, it has been shown convincingly in the NOD model of diabetes that low affinity epitopes exist and are capable of transferring disease (Levisetti et al. 2008). Various reports have observed that DR1201 influences the development of various forms of inflammatory arthritis (Cerna et al. 1994; Liao et al. 2007; Albert and Scholz 1998) and sarcoidosis (Grunewald 2010). Our results indicate that low affinity epitopes (including H1HA₁₈₇₋₂₀₃ and H1NP₁₀₉₋₁₂₅) exist even in the context of influenza responses.

However, T cells with these specificities were readily detected using tetramers. Therefore, it is likely that T cell responses in autoimmune settings such as arthritis and sarcoidosis are still amenable to study using these HLA specific tools and methodologies.

5 Conclusions

Using a combination of tetramer guided epitope mapping, peptide competition assays and molecular modeling, this study characterized the peptide-binding properties of HLA-DRB1*1201 (DR1201). The observed motif had distinct features, collectively not seen in any single MHC II allele thus far, including a preference for aliphatic anchor residues at pocket 1 and an unusually restrictive pocket 4. Pocket 6 was extremely permissive, while pocket 9 preferred bulky, polar, and acidic residues, but excluding basic residues. This observed DR1201 binding motif was in agreement with 14 of 15 novel 20mer peptides identified by tetramer guided epitope mapping as DR1201 epitopes and consistent with the measured binding of these peptides to DR1201 protein. These findings should aid in the understanding and prediction of DR1201 restricted T cell epitopes for autoimmune

responses in the context of inflammatory arthritis and sarcoidosis and in protective immune responses against Hepatitis antigens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by NIH contract HHSN266200400028C.

Abbreviations

DR1201	DRB1*12:01
DR1104	DRB1*11:04
H1HA	Influenza A/New Caledonia/2-/99 Hemagglutinin
H1MP	Influenza A/Puerto Rico/8/34 Matrix Protein M1
H1NP	Influenza A/Puerto Rico/8/34 Nucleoprotein
TTL	Tetanus Toxin Light Chain
TT	Tetanus Toxin Heavy Chain
RBA	Relative binding affinity

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Highlights

- ■ We characterize the binding motif of DR1201, an HLA-DR5 allele that lacks β 57Asp
- ■ β 9Glu/ β 30His/ β 37Leu/ β 57Val create a spacious pocket 9 that prefers aromatics
- ■ The preferences of DR1201 are distinct from the closely-related DR1104 allele
- ■ These preferences may lead to the selection of a unique T cell repertoire

Figure 1.

Tetramer Guided Epitope Mapping of Influenza matrix protein epitopes. (A) Pool Mapping: T cells from a DR1201 donor were stimulated with overlapping peptide mixtures spanning H1MP and stained using pooled peptide loaded tetramers after two weeks. Peptide pools 1 and 2 gave positive staining. (B) Individual peptide mapping: T cells from the tetramer positive panels shown in A were stained again using tetramers loaded with individual peptide from the corresponding peptide pools. Peptides MP₃₃₋₅₂ and MP₄₁₋₆₀ were identified as peptides containing DR1201 restricted epitopes.

Figure 2.

Binding register of the test peptide. Observed relative binding affinities for derivatives of the MP₄₀₋₅₄ peptide with non-conservative substitutions are shown. Relative binding affinity (RBA) values were calculated as the IC₅₀ value of the un-substituted peptide divided by the IC₅₀ value of the substituted peptide. Values are shown on a logarithmic scale centered on 0.01

Figure 3.

Amino acid preferences for A) pocket 1, B) pocket 4, C) pocket 6, and D) pocket 9 of the DR1201 motif. Black bars represent values within 2.5-fold of the reference peptide (RBA 0.4, “preferred”), hatched bars represent values within 10-fold of the reference peptide (RBA 0.05, “tolerated”), and open bars represent values within less than 20-fold of the reference peptide (RBA 0.05, “excluded”).

Figure 4.

(A) T cell receptor view of the antigenic peptide (VLMEWLKTRPILS, anchors in bold) in the groove of DR1201. The $\alpha 1\beta 1$ domain of the DR12 molecule is depicted in van der Waals surface representation, with the surface atomic charges color-coded (blue, positive; grey, neutral; red, negative), and the antigenic peptide is shown in space filling form (color code: carbon, green; oxygen, red; nitrogen, blue; hydrogen, white; sulfur, yellow). Several DR12 residues that have particular interactions with the antigenic peptide ($\alpha 54$ Phe, $\alpha 58$ Gly, $\alpha 62$ Asn, $\alpha 65$ Val, $\alpha 72$ Ile, $\alpha 73$ Met, $\alpha 76$ Arg, $\beta 57$ Asp, $\beta 60$ Ser, $\beta 61$ Trp, $\beta 66$ Asp, $\beta 67$ Ile, $\beta 70$ Asp, $\beta 71$ Arg, $\beta 74$ Ala, $\beta 78$ Tyr) are shown in stick form with the same color-code as the antigenic peptide with the exception of carbon (orange). (B) TCR view of pockets 4 and 6 of the DR1201/VLMEWLKTRPILS complex (substituted to depict Phe in pocket 6). In pocket 4 hydrophobic residues form the walls of the pocket and charged residues ($\beta 70$ Asp, $\beta 71$ Arg) move out of the pocket. Pocket 6 is essentially composed of hydrophilic residues, except $\beta 11$ Ser, but the ample room left by $\beta 13$ Gly allows for this arrangement. Depiction and color conventions are identical to Fig. 4A. (C) TCR view of pocket 9 of the DR1201/VLMEWLKTRPILS complex (substituted to depict the accommodation of Phe). The several hydrophobic and mostly flexible residues in this pocket ($\alpha 72$ Ile, $\alpha 73$ Met, $\alpha 37$ Leu, $\beta 57$ Val, $\beta 61$ Trp), permit Tyr/Phe to fit exquisitely. Depiction and color conventions are identical to Fig. 4A.

DRB1*1201 Peptide Binding Motif									DRB1*1104 Peptide Binding Motif								
Position									Position								
1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9
I			L		A			Y	I			L		R			A
L			I		M			F	L			V		K			G
M			(M)		F			M	V			M		H			S
F			(K)		Q			I	A			F					P
V			(R)		V			V	Y								
					S			(Q)									
					T			(E)									
					L			(D)									
					E			(S)									
					N			(N)									
					Y												
					(P)												
					(H)												
					(K)												
					(R)												

Figure 5.

Comparison of the amino acid preferences for DR1201, as determined by the present study and DR1104 as reported by Verreck et al. (1996). For both alleles preferred residues are bolded. For DR1201 tolerated residues are shown in round brackets.

Table I

Sequences and Predicted Motifs of Novel and Published DR1201 Epitopes

Peptide	Sequence ^a	Pred RBA	Meas RBA
H1HA ^b 181-197	YVNNKEKE VLVLWGVHH	0.10	0.05
H1HA ^b 187-203	KE VLVLWGVHH PPNIGN	0.10	0.02
H1MP ^c 33-52	AGKNTDLEVL MEWLKTRPIL	1.0	1.0
H1MP ^c 41-60	VL MEWLKTRPIL SPLTKGIL	1.0	0.97
H1NP ^d 109-125	VL YDKEEIRRIWRQANN	0.02	0.01
H1NP ^d 408-424	TQPT FSVQRNL PFDKTT	0.13	0.75
TTL ^e 241-260	GLYGMQVSSHE IIPSKQEIY	1.5	1.7
TTL ^e 249-268	SHE IIPSKQEIY MQHTYPIS	1.5	2.8
TTL ^e 257-276	QEIYMQHTYPISAEEL FTFG	1.1	3.3
TTL ^e 281-300	NLSIDIKNDL YEKTLNDYK	2.2	1.9
TT ^f 602-621	KVNQGAQ GILFLQWVRDIID	0.10	0.36
TT ^f 746-765	EYQVDA IKKIIDYEYKIYSG	0.74	0.79
TT ^f 1050-1069	GVLMGSAEIT GLGAIRE DNN	3.4	0.70
TT ^f 1234-1253	IPLYKKMEAV KLRDLKTYSV	3.4	2.1
TT ^f 1242-1261	AVKLRDLKTYSV QLKLYDDK	3.4	1.9
HCVPP ^g 1248-1261	GYKVLVLNPSVAAT	0.01	
HBsAg ^h 241-258	PICPGYRWM MCLRRFIIFL	1.5	
HBsAg ^h 341-357	SVRF SWLSLLVPFVQWF	0.71	
H3HA ⁱ 305-328	CPKYVKQNTL KLATGMRNVPEKQT	0.01	
EBNA-1 ^j 561-573	YFM VLQTHIFAE	14.0	
MVNP ^k 229-243	LRRFMVALIL DIKRT	0.04	

^aPredicted binding registers indicated in boldface, secondary registers underlined^bH1HA: Influenza A/New Caledonia/20/99 Hemagglutinin^cH1MP: Influenza A/Puerto Rico/8/34 Matrix Protein M1^dH1NP: Influenza A/Puerto Rico/8/34 Nucleoprotein^eTTL: Tetanus Light Chain^fTT: Tetanus Toxin Heavy Chain^gHCVPP: Hepatitis C virus polyprotein (Diepolder et al. 1997)^hHBsAg: Hepatitis B Virus surface antigen (Desombere et al. 2000)ⁱH3HA: Influenza A/Texas/1/1977 Hemagglutinin (Zeliszewski et al. 1994)^jEBNA-1: Epstein-Barr virus-encoded nuclear antigen 1 (Voo et al. 2005)

k MVNP: Measles virus nucleocapsid protein (Marttila et al. 1999)

Table II**Relative Binding of Substituted influenza Peptides**

Sequence	Substitution	RBA ^a
EVLMEWLKTRPILS	Unmodified	1.0
EVL Y EWLKTRPILS	P1 M-->Y	0.02
EVL F EWLKTRPILS	P1 M-->F	0.73
EVL L EWLKTRPILS	P1 M-->L	1.5
EVL I EWLKTRPILS	P1 M-->I	2.2
EVL V EWLKTRPILS	P1 M-->V	0.64
EVL A EWLKTRPILS	P1 M-->A	0.02
EVL K EWLKTRPILS	P1 M-->K	0.02
EVL R EWLKTRPILS	P1 M-->R	0.02
EVLMEW Y KTRPILS	P4 L-->Y	0.02
EVLMEW F KTRPILS	P4 L-->F	0.02
EVLMEW I KTRPILS	P4 L-->I	0.31
EVLMEW P KTRPILS	P4 L-->P	0.02
EVLMEW M KTRPILS	P4 L-->M	0.15
EVLMEW V KTRPILS	P4 L-->V	0.04
EVLMEW A KTRPILS	P4 L-->A	0.02
EVLMEW D KTRPILS	P4 L-->D	0.02
EVLMEW E KTRPILS	P4 L-->E	0.02
EVLMEW Q KTRPILS	P4 L-->Q	0.02
EVLMEW N KTRPILS	P4 L-->N	0.02
EVLMEW S KTRPILS	P4 L-->S	0.02
EVLMEW H KTRPILS	P4 L-->H	0.02
EVLMEW K KTRPILS	P4 L-->K	0.10
EVLMEW R KTRPILS	P4 L-->R	0.05
EVLMEWLK S RPILS	P6 T-->S	1.3
EVLMEWLK N RPILS	P6 T-->N	0.61
EVLMEWLK Q RPILS	P6 T-->Q	1.6
EVLMEWLK Y RPILS	P6 T-->Y	0.46
EVLMEWLK F RPILS	P6 T-->F	2.1
EVLMEWLK L RPILS	P6 T-->L	0.70
EVLMEWLK H RPILS	P6 T-->H	0.32
EVLMEWLK M RPILS	P6 T-->M	2.7
EVLMEWLK V RPILS	P6 T-->V	1.3
EVLMEWLK A RPILS	P6 T-->A	5.1
EVLMEWLK P RPILS	P6 T-->P	0.37
EVLMEWLK K RPILS	P6 T-->K	0.20

Sequence	Substitution	RBA ^a
EVLMEWLKERPILS	P6 T-->E	0.63
EVLMEWLKDRPILS	P6 T-->D	0.02
EVLMEWLKRRPILS	P6 T-->R	0.15
EVLMEWLKTRPALS	P9 I-->A	0.02
EVLMEWLKTRPVLS	P9 I-->V	0.75
EVLMEWLKTRPMLS	P9 I-->M	2.9
EVLMEWLKTRPFLS	P9 I-->F	14.0
EVLMEWLKTRPYLS	P9 I-->Y	18.0
EVLMEWLKTRPHLS	P9 I-->H	0.04
EVLMEWLKTRPPLS	P9 I-->P	0.02
EVLMEWLKTRPQLS	P9 I-->Q	0.35
EVLMEWLKTRPNLS	P9 I-->N	0.11
EVLMEWLKTRPSLS	P9 I-->S	0.12
EVLMEWLKTRPDLS	P9 I-->D	0.12
EVLMEWLKTRPELS	P9 I-->E	0.30
EVLMEWLKTRPKLS	P9 I-->K	0.02
EVLMEWLKTRPRLS	P9 I-->R	0.02

^aIC₅₀ of un-substituted peptide is 0.7 μM

Table III

Differences in residues for pockets 6, 7 and 9 among DRB1*1104, DRB1*1201 and DQB1*0604

β -chain residue	DRB1*1104	DRB1*1201	DQ0604	pocket
9	Glu	Glu	Tyr	9
16	His	Tyr	Tyr	---
26	Phe	Leu	Leu	4
28	Asp	Glu	Thr	7
30	Tyr	His	His	6
32	Tyr	His ^a	Tyr	---
37	Tyr	Leu	Tyr	9
38	Val	Leu	Ala	---
47	Phe	Phe	Tyr	7
57	Asp	Val	Val	9
58	Glu	Ala	Ala	---
60	Tyr	Ser	Tyr	---
67	Phe	Ile	Val	7
71	Arg	Arg	Thr	7

^aWhile β 32His is not part of any pocket, its proximity to β 9Glu would probably cause in the endosomal pH of 5.5, the former residue to remain positively charged and in proximity to the latter; this association and interaction could be maintained also while DRB1*1201 would be on the cell membrane, if the residues at pockets 6 and 9 would not be in any way antagonistic to it.