An example of immunodominance: engagement of synonymous TCR by invariant CDR3β

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Abstract

The structural basis of the T cell response against immunodominant tetanus toxin (TT)-derived peptides was investigated using TT-specific T cell clones raised from a DRB1*0301 homozygous donor. Three peptides forming T cell epitopes were identified, including one, TT(1272–1284), that stimulated four different TT-specific T cell clones. TCR sequence analysis revealed that these synonymous TCR shared only arginine at the third position of the CDR3β loop. This prominent residue may form a salt bridge with a corresponding aspartate at the relative position 8 (P8) of the antigenic peptide TT(1272–1284) as suggested from amino acid replacement analysis. A similar scenario was observed for a second TT epitope, TT(279–296), and its corresponding TCR. These examples show that immunodominance may result from a single strong amino acid interaction between TCR CDR3β loops here in contact with the C-terminus of the antigenic peptide. Such a dominant interaction could compensate for weaker contacts between other residues of the TCR and the antigenic peptide, and would allow the recognition of a single peptide–MHC complex by a broader synonymous TCR repertoire and could thus contribute to its immunodominance.

Introduction

T lymphocytes recognize a complex of antigenic fragments associated with MHC on antigen-presenting cells (APC) (1,2). The antigen-specific αβ heterodimers of the TCR contain hypervariable regions termed complementarity-determining regions (CDR) like the corresponding segments of Ig. CDR1 and CDR2 sections are encoded by the variable gene segments of TCR α and β genes, and their contribution to diversity is rather low. By contrast, CDR3α and CDR3β regions are created by somatic rearrangement of variable (Vα) and joining (Jα) or Vβ and diversity (Dβ) and Jβ gene segments. While the TCR CDR3α and CDR3β loops seem to contact mainly residues of the MHC molecules (1,3), although it has been described that in the 2C TCR–dEV8 peptide–MHC H-2Kb complex these loops interacted with the peptide residues simultaneously (4).

Regardless whether the T cell activation is initiated by conformational changes or by cross-linking of multiple TCR complexes on the surface of the T cell, it is the quality of these interactions that affects the quality of the T cell response. It has been shown that the affinities of a TCR bound to a MHC class II molecule with agonistic or antagonistic peptide ligands strictly correlate with the responsiveness of the T cell (5). In addition, modest variations in the affinity of MHC class I molecules with positively or negatively selecting ligands for the same TCR are capable of triggering different signals (6). A strong T cell response is usually seen when immunodominant peptides are bound to the restricting MHC. The phenomenon of immunodominance in the class II system is mainly thought to be caused by successful peptide competition for MHC. The phenomenon of immunodominance in the class II system is mainly thought to be caused by successful peptide competition for MHC class II binding in the loading compartment of APC (7). Alternatively, immunodominance could be explained by different antigen-processing mechanisms in different APC as it has been recently reported (8). To explore another attractive possibility, we investigated the structural features of the interaction of immunodominant epitopes derived from...
the model antigen tetanus toxin (TT) with their corresponding TCR. These epitopes triggered strong responses in several TT-specific T cell clones from DRB1*0301/DRB3*0101 donors. To understand how the hierarchy of immunodominance within a pool of similarly active antigenic peptide derived from the same protein may be established, we identified the residues in each peptide that interact with the corresponding TCR. Based on the previously reported crystal structure data of the DRB1*0301 complexed with the CLIP peptide (9) we predicted that, within the nonamer core segments fitting the groove, peptide residues at positions (P) P2, P5 and P8 projected out of the MHC class II binding groove facing the TCR. Hence, we focused our attention on these residues in examining the structural basis of antigen recognition of a panel of dominant TT epitopes. In these cases, we present evidence for a critical role of residue P8 of the nonamer core segment for the stimulation of TT-specific T cells. Moreover, the majority of TCR examined showed a strong interaction between a particular CDR3β residue (in our case no. 3) and the corresponding residue at P8 of the peptide. In the context of the DRB1*0301/DRB3*0101 haplotype, this interaction enables the stimulation of at least four different synonymous TCR by the major T cell epitope TT(1272–1284).

Methods

Generation of antigen-specific T cell lines

Antigen-specific T cell lines were raised from a homozygous DRB1*0301/DRB3*0101-positive donor as described (10). All blood samples were obtained after informed consent. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood or buffy coats by density-gradient centrifugation and stimulated with TT (1–5 μg/ml; Calbiochem, La Jolla, CA) in culture medium RPMI 1640 (Gibco BRL, Karlsruhe, Germany) supplemented with 3% pooled human serum (blood bank), 2 mM glutamine and antibiotics (Gibco). Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood or buffy coats by density-gradient centrifugation and stimulated with TT (1–5 μg/ml; Calbiochem, La Jolla, CA) in culture medium RPMI 1640 (Gibco BRL, Karlsruhe, Germany) supplemented with 3% pooled human serum (blood bank), 2 mM glutamine and antibiotics (Gibco). T cells were assayed for antigen and peptide specificity after 6 days in primary culture as well as after periodic re-stimulation with TT and autologous irradiated (30 Gy) APC. Briefly, 10^4 TT-specific T cells were co-cultured with 10^5 irradiated (3000 rad) autologous PBMC in duplicates or triplicates respectively with and without antigens and peptides (10 μg/ml or as indicated). After 48 h, the cultures were labeled with 0.5 μCi/well [3H]thymidine (sp. act. 5 μCi/mM; Amersham-Buchler, Braunschweig, Germany) and the radioactivity incorporated was measured 16 h later by liquid scintillation counting.

T cell clones

TT-specific T cells were cloned by limiting dilution in microtiter plates using 0.3 cells/well, together with 2×10^4 irradiated autologous PBMC preincubated with TT in complete medium containing 20 U/ml of IL-2 as described (10) and maintained in culture by periodic re-stimulation with autologous APC and TT. The T cell clones Pil-1, Pil-5, Pil-6 and Pil-36 [TT(1272–1284)], Pil-2 and Pil-33 [TT(1061–1075)] and Pil-45 [TT(279–296)] were obtained from the same DRB1*0301/DRB3*0101 homozygous donor (Pil).

Antigens

Native TT was obtained from Calbiochem (La Jolla, CA) and used in 0.01–10 μg/ml final concentrations. This holotoxin preparation is no longer distributed by Calbiochem. The cDNA encoding amino acids 865–1315 of TT (TT C-fragment, kindly provided by H. Niemann) was cloned and engineered to contain a C-terminal histidine-tag, which allows metallo-affinity chromatography on Ni2+ resins. Single colonies of transformed Escherichia coli M15 were grown in 500 ml 2×YT medium with 50 μM ampicillin and 25 μM kanamycin to an OD_{600} of 0.6–1.0, and were induced with 0.5 mM isopropyl-thiogalacto-pyranoside. After 2–4 h, the bacteria were harvested by centrifugation (3000 g for 20 min at 4°C). The pellet was resuspended in 50 mM phosphate buffer, 300 mM NaCl and 0.1 mM PMSF, pH 8.0. Bacteria were lysed in the same buffer containing 600 mM NaCl by three cycles of freezing and thawing, followed by three cycles of sonification with a Branson sonifier at 4°C for 30 s each. The lysate was centrifuged at 36,000 g and the supernatant passed over 1 ml Ni-NTA agarose (Qiagen, Hilden, Germany). The beads were washed with lysis buffer at pH 8.0 and 6.2, and eluted with 50 mM NaH_2PO_4, pH 4.5.

Peptides

Peptides (Table 1) were synthesized in an automated peptide synthesizer 432A (Applied Biosystems, Weiterstadt, Germany) following the Fmoc/tBu strategy, and analyzed by HPLC (System Gold; Beckmann Instruments, München, Germany) and MALDI-TOF mass spectrometry (G2025A; Hewlett-Packard, Waldbronn, Germany). Peptides showing a purity of <80% were purified by preparative HPLC. N-terminal biotinylation of apoB100(2877–2894) was carried out by five coupling steps using Fmoc-β-alanine, Fmoc-ε-aminocaproic acid (Ahx), Fmoc-ε-lysine and Dmtr-biotin to form biotin-ε-Lys-β-Ala-Ahx-β-Ala-[apoB100(2877–2894)].

DRB1*0301 (DR17) purification

HLA-DR molecules were purified from the DRB1*0301 (DR17)-transfected T2.DR3 cells (expressing only DRB1*0301) using mAb L243- (11,12) coupled CNBr-activated Sepharose (Pharmacia, Freiburg, Germany) as described previously (13). DRB1*0301 molecules were eluted with 25 mM Na_2CO_3, 0.15 M NaCl, 0.1% NP-40 and 0.1 mM PMSF, pH 11.0, neutralized immediately with 50 mM Tris–HCl, pH 8.4, and stored at 4°C until use. The presence of intact DRB1*0301 molecules was confirmed by PAGE under non-reducing conditions (not shown).

Peptide binding assay

Purified DRB1*0301 molecules (100 nM) were incubated at 37°C with 2 μM of the biotinylated, high-affinity DR17 ligand apoB(2877–2894) with or without competitor peptide in 96-well microtiter plates (Greiner, Nütingen, Germany) in binding buffer (50 μl) containing 2 mM EDTA, 0.01% azide, 0.1 mM PMSF and 0.1% NP-40 adjusted to pH 5.0 by 1 M citrate. After 72 h, the neutralized sample of MHC–peptide complexes was separated from peptide excess by immunoprecipitation, and detected by successive incubation at 20°C with streptavidin (2.5 μg/ml) (Dianova, Hamburg, Germany) and bio-
for 10 min, for 1 cycle. Primer sequences were as follows

Intact TT protein in subnanomolar concentrations induced a

precipitation, the purification of single-strand cDNA by ammonium acetate pre-

nable cDNA was ligated to a 3′-NH2 blocked In order to study the molecular interaction in the recognition

Results

DRB1*0301 (DR17) motif peptides stimulate TT-specific T cell clones: correlation with naturally processed T cell epitopes

In order to study the molecular interaction in the recognition of antigenic peptides by class II-restricted T cells, we choose TT as a model antigen and evaluated the recognition pattern of a set of TT peptides predicted to bind to DRB1*0301 (DR17) molecules due to the presence of the DR17-binding motif (13,18,19). We raised T cell clones against the TT protein and used them as reporter cells to confirm that the preselected TT-peptides are indeed generated by autologous APC. T cell conditions were used for the

Table 1. Binding capacity of TT-derived peptides and variants to DRB1*0301 (DR 17)

<table>
<thead>
<tr>
<th>DRB1*0301 (DR 17) peptides and variants</th>
<th>Relative positiona</th>
<th>Concentration giving 50% inhibition (µM)b</th>
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</thead>
<tbody>
<tr>
<td>TT(279–296)</td>
<td>D A N L I S I D I K N D L Y E K T L</td>
<td>0.8</td>
</tr>
<tr>
<td>TT(279–296) D→L</td>
<td>D A N L I S I D I K N L L Y E K T L</td>
<td>1.0</td>
</tr>
<tr>
<td>TT(279–296) D→K</td>
<td>D A N L I S I D I K N K L Y E K T L</td>
<td>2.2</td>
</tr>
<tr>
<td>TT(279–296) D→E</td>
<td>D A N L I S I D I K N E L Y E K T L</td>
<td>1.1</td>
</tr>
<tr>
<td>TT(1061–1075)</td>
<td>L G A I R E D N N I T L K L D</td>
<td>4.0</td>
</tr>
<tr>
<td>TT(1272–1284)</td>
<td>N G Q I G N D P N R D I L</td>
<td>84.0</td>
</tr>
<tr>
<td>TT(1272–1284) D→L</td>
<td>N G Q I G N D P N R L I L</td>
<td>13.0</td>
</tr>
<tr>
<td>TT(1272–1284) D→K</td>
<td>N G Q I G N D P N R K I L</td>
<td>26.0</td>
</tr>
<tr>
<td>TT(1272–1284)</td>
<td>N G Q I G N D P N R E I L</td>
<td>42.0</td>
</tr>
</tbody>
</table>

aConserved anchor residues are indicated in bold whereas exchanged residues of antigenic peptide position P8 are underlined.

bBinding data are expressed in terms of relative binding capacity and represent mean values of two independent experiments.

tinylated peroxidase (100 ng/ml; Dianova) for 45 min respectively, followed by incubation with ABTS (1 mg/ml) (Boehringer Mannheim, Mannheim, Germany). The absorbance at 405 nM was measured by an ELISA reader (Multiskan Plus; TiterTek, Meckenheim, Germany) and non-specific signals (quadruplicates, typically <15% of maximal absorbance) were subtracted from the data.

Measurement of MH C class II binding of peptide variant mixture P8 was performed using the europium fluoroimmunoassay in a Wallac 1420 Victor multilabel counter (Wallac-ADL, Freiburg, Germany) as described (14).

Cloning and sequence determination of TCR

Cloning of the 5′ end of TCR cDNAs, including the CDR3 and a part of the constant region, was performed by ligation-anchored PCR as described elsewhere (15). Briefly, total RNA was isolated using RNeasy (Qiagen, Valencia, CA) and oligo(dT) (Pharmacia, Freiburg, Germany) as primer. After first-strand cDNA synthesis, the RNA template was removed by treatment with sodium hydroxide (0.3 M) at 50°C for 30 min. Following neutralization with acetic acid and purification of single-strand cDNA by ammonium acetate precipitation, the purified cDNA was digested to a 3′-NH2 blocked and 5′-phosphorylated anchor oligonucleotide (15) by T4 RNA ligase (New England Biolabs, Beverly, MA). The anchored cDNA was amplified by a first PCR reaction using a primer complementary to the anchor (F8/26) and one from the TCRα or TCRβ constant region, near the 3′ end. These primers were used at a final concentration of 0.06 µM (16). The following conditions were used for the first PCR: 94°C for 4 min, for 1 cycle; 94°C for 40 s, 56°C for 40 s, 72°C for 90 s, for 14 cycles; and 72°C for 10 min, for 1 cycle.

The diluted first-round PCR products were used as a template (final dilution 1:100–1:400) for a nested PCR with same forward primer and inside reverse primers from the TCRα or TCRβ constant region. The following conditions were used for the second, nested PCR: 94°C for 4 min, for 1 cycle; 94°C for 40 s, 56°C for 40 s, 72°C for 90 s, for 35 cycles; and 72°C for 10 min, for 1 cycle. Primer sequences were as follows

(anchor: CTGCATCTAATGCTCCTCTCGCTACCTGTC-
ACTCTGCGTGACATC; forward primer: F8/26: CGCAGA-
GTGACGGTAGTAC; reverse primers: RA-1: GAGGAAAG-
GAGCAGGAGGAG; RA-2: GTACAGGGCCAGGTCCAGG; RB-
1: GAGGAGCATCTGGAGTCATTG; RB-2: TCTGAGGCCT-
CAAACACAGC). PCR amplification of TCR cDNA using a panel of Vα and Vβ subfamily-specific primers was performed as previously described (17). PCR products were polished by Pfu DNA polymerase (Stratagene) for blunt-end ligation into a Smal-digested pBluescript KS+ vector (Stratagene). Sequence analysis was performed using the Amplitaq FS dye deoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, California) and read in a PE-ABI Prism 310 automated DNA sequencer.

The entire 5′ ends of TCR cDNAs from TT-specific T cell clones, except the β chain of TCR from clone Pil-6, were sequenced after cloning of the 5′-anchored PCR products. The TCR β sequence of clone Pil-6 was obtained after using the Vβ subfamily-specific primer panel described above.

Results

DRB1*0301 (DR17) motif peptides stimulate TT-specific T cell clones: correlation with naturally processed T cell epitopes

In order to study the molecular interaction in the recognition of antigenic peptides by class II-restricted T cells, we choose TT as a model antigen and evaluated the recognition pattern of a set of TT peptides predicted to bind to DRB1*0301 (DR17) molecules due to the presence of the DR17-binding motif (13,18,19). We raised T cell clones against the TT protein and used them as reporter cells to confirm that the preselected TT-peptides are indeed generated by autologous APC. T cell clones Pil-1, Pil-4, Pil-5, Pil-6 and Pil-36 recognized the TT(1061–1284) sequence, which is similar to the previously described TT(1061–1284) by Demotz et al. (20). T cell clone Pil-2, previously designated PK-1 (21), and Pil-33 from this donor recognized a new determinant TT(1061–1075), and 0.5 nM of this peptide was sufficient to induce a strong proliferative response (stimulation index > 10, Fig. 2). Intact TT protein in subnanomolar concentrations induced a similar strong proliferation of Pil-2 (Fig. 2), indicating that
Engagement of synonymous TCR by an immunodominant epitope

Fig. 1. Proliferative response of TT(1272–1284)-specific T cell clones stimulated with native TT protein (1 µg/ml, open bars) or without antigenic stimulation (filled bars). Cloned T cells (1×10^5) were stimulated with 1×10^6 autologous irradiated (30 Gy) PBMC per well and antigenic protein as indicated. Cells were labeled after 48–60 h with 0.5 µCi [3H]thymidine/well and harvested 16 h later. Proliferation was determined by the incorporation of [3H]thymidine.

Fig. 2. Fine specificity of T cell clone Pil-2 raised from limiting dilution. Processing of the T cell epitope TT(1061–1075) was confirmed with native TT and recombinant TT C-fragment. Background value was 1557 ± 71 c.p.m.

TT(1061–1075) was efficiently processed in autologous APC from the native TT protein. Pil-45 cells responded to nanomolar concentrations of TT(279–296) (Fig. 3), confirming that all these motif peptides were also equivalents of naturally processed T cell epitopes derived from TT protein.

Binding of CD4+ T cell-stimulating TT-derived peptides to DRB1*0301

To investigate the restriction of the immunogenic TT-derived motif peptides we examined their binding to the DRB1*0301 molecules which were purified from T2.DR3 cells (22). The binding to DRB1*0301 molecules was analyzed at endosomal pH 5 in competition with the biotinylated natural ligand apoB(2877–2894) (13,14,19). The binding capacity was defined as the molar concentration of unlabeled TT peptides required for 50% inhibition of 2 µM biotinylated agonist binding (= IC50; Table 1). While TT(279–296) (IC50 0.8 µM) and TT(1061–1075) (IC50 4.0 µM) showed excellent binding capacity, we measured a moderate binding of TT(1272–1284) to DRB1*0301 molecules (IC50 84.0 µM) in vitro. This argues that TT(279–296) and TT(1061–1075) are presented by DRB1*0301, whereas TT(1272–1284) could be cross-presented both by DRB1*0301(DR17) and the simultaneously expressed DRB3*0101 (DR52a) as reported previously (19,23).

Structural prediction of antigenic peptide conformation bound to DRB1*0301 and DRB3*0101—identification of potential TCR contact residues

To further investigate the structural basis of the strong T cell responses to those TT-derived antigenic peptides in the context of DRB1*0301/DRB3*0101, we analyzed their theoretical conformation based on the crystal structure data of the DRB1*0301–CLIP peptide complex (9). Interestingly, this class II molecule was purified from a B lymphoblastoid cell line (9.5.3) which effectively presented TT(1274–1285) to DRB1*0301 molecules (IC50 84.0 µM) in vitro. This argues that TT(279–296) and TT(1061–1075) are presented by DRB1*0301(DR17) and the simultaneously expressed DRB3*0101 (DR52a) as reported previously (19,23).
Engagement of synonymous TCR by an immunodominant epitope

Fig. 4. Proposed model of the invariant Arg–Asp interaction between HLA-DRB1*0301-restricted TCR and TT-derived antigenic peptides. The arrows under peptide residues P1, P4, P6 and P9 indicate contact sites/anchors with the MHC class II molecule; the arrows pointing towards the TCR highlight potential TCR contact sites. See Results and Discussion for details.

class II complexes suggested a common polyproline II-like conformation of the bound antigenic peptides. (26,27). With regard to the highly efficient binding of the epitopes TT(279–296) and TT(1061–1075) peptides to DRB1*0301 (Table 1), it can be argued that these peptides compete very efficiently for binding to the DRB1*0301 heterodimer in the antigen loading compartments of APC. Both peptides should therefore be presented preferentially on the DRB1*0301 molecule. Analysis of the conformation of CLIP bound to DRB1*0301 allowed a predicted alignment of the three TT-derived epitopes (Fig. 4). Isoleucine at the N-terminal position 1 in all TT peptides should contact the non-polar pocket 1 of DRB1*0301 whereas the C-terminal leucine of TT(279–296) and TT(1061–1075) and the isoleucine of TT(1272–1284) may be deeply buried in pocket 9. Slight movements of DRB1*0301 side chain β74R in pocket 4 should allow the fitting of the DRB1*0301-allele specific anchor residue aspartate in this pocket (9). Finally, the anchor residue P6, which is lysine in TT(279–296) and asparagine in TT(1061–1075) and TT(1272–1284), correlates well to the known DRB1*0301 binding motif as well as to the negatively charged binding pocket 6. Knowing the anchor residues of the TT-derived peptides and the X-ray crystal structure of the CLIP-DRB1*0301 complex we were also able to predict potential TCR contact residues. Up to date all published crystallographic data of MHC class II–peptide complexes reveal a highly conserved conformation of the peptides in the binding groove (26,27). Thus, we suggest that the residues P1, P2, P5 and P8 should be primarily exposed to the TCR (Fig. 4).

TCR syntax: sequence analysis of TT-specific TCR

To gain insight into the essential process of peptide recognition by T cells, sequence information of antigen contact sites in antigen-specific T cell receptors is required. As shown in Table 2, in the case of the independently raised clones Pil-2 and Pil-33 [specific for TT(1061–1075)], as well as clones Pil-1 and Pil-5 [specific for TT(1272–1284)], each pair has identical TCR sequences at the nucleotide level in their α or β chains, indicating that they can be traced back to same founders. Interestingly, TT(1272–1284), a prominent T cell epitope already described (20), was recognized by four different T cell clones (Pil-1, Pil-4, Pil-6 and Pil-36; see Figs 1 and 4, and Table 2). All four clones used distinct CDR3α and CDR3β sequences at the amino acid level (see Table 2). In particular, Pil-4 cells expressed two productively rearranged TCR α subunits, as has been described previously within a fraction of CD4+ T cells (28–30). One α subunit belonged to the TCR gene family TCRADV21S1A1N, whereas the other α
chain was identified as TCRAV1S3A1T (31). Since another TT(1272–1284)-specific T cell clone, Pil-36, used the identical TCRAV21S1A1N gene segment as Pil-4, we conclude that TCRAV21S1A1N mediates antigen recognition in Pil-4 cells rather than the TCRAV1S3A1T segment. In addition, both T cell clones Pil-1, Pil-36, Pil-4 and Pil-6 were highly responsive to the wild-type TT(1272–1284) ligand as well as to the previously described shorter variant TT(1273–1284) (20), whereas the exchange of the negatively charged residue aspartate at position P8 of the TT(1273–1284) sequence from T cell clones Pil-4 and Pil-36 express the same V\textsubscript{\textalpha} and V\textsubscript{\textbeta} gene segments for CDR1 and CDR2, although the junctional regions, including CDR3\textalpha and CDR3\textbeta, are quite different. Regarding TCR pairing and determination of antigen specificity, we noted that TCR \textalpha chains of the clones Pil-4 and Pil-2/Pil-33 (examined in independent experiments) showed absolute sequence identity, including their hypervariable CDR3\textalpha region, but differed entirely in their \textbeta chain usage, and they recognize different antigen peptides, TT(1061–1075) and TT(1272–1284) respectively (see Table 2). As shown in Fig. 5, TT(1272–1284)-specific T cell clones Pil-1, Pil-36, Pil-4 and Pil-6 were highly responsive to the wild-type peptide as well as to the previously described shorter variant TT(1273–1284) (20), whereas the exchange of the negatively charged residue aspartate at position P8 of the TT(1272–1284) peptide variants was tested, a similar, although not consistent, effect was observed by introducing an inversely charged residue (lysine for aspartate) which further reduced the T cell response activity of at least one order of magnitude. Table 2, all TCR V\textsubscript{\textalpha} sequences from T cell clones Pil-1, Pil-4, Pil-6 and Pil-36 responding to TT(1272–1284) and Pil-45, which recognized another TT peptide TT(279–296), contained arginine at the same relative position of the CDR3\textbeta loop. On the other hand, all antigenic peptides that are recognized by these clones had aspartate at relative position P8 (see Table 1). This consistent finding is a strong argument for the formation of a salt bridge with the arginine residue of the CDR3\textbeta loops. Strong interactions between the CDR3\textbeta loops of TCR and position P8 of the corresponding antigen peptide were demonstrated earlier by Sant’Angelo et al. (32).

**Table 1. TCR sequence analysis of TT-specific T cell clones**

<table>
<thead>
<tr>
<th>T cell clone</th>
<th>TCR V\textsubscript{\textalpha}</th>
<th>TCR V\textsubscript{\textalpha} junctional sequence</th>
<th>Peptide specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pil-1-2/33</td>
<td>ADV21S1A1N</td>
<td>CAAS GAGNNRKLI WGLGTSLAVNPN</td>
<td>TT(1061–1075)</td>
</tr>
<tr>
<td>Pil-1-4</td>
<td>ADV21S1A1N</td>
<td>CAAS GAGNNRKLI WGLGTSLAVNPN</td>
<td>TT(1272–1284)</td>
</tr>
<tr>
<td>Pil-1-36</td>
<td>ADV21S1A1N</td>
<td>CAAS GAGNNRKLI WGLGTSLAVNPN</td>
<td>TT(1272–1284)</td>
</tr>
<tr>
<td>Pil-1-5</td>
<td>ADV21S1A1N</td>
<td>CAAS GAGNNRKLI WGLGTSLAVNPN</td>
<td>TT(1272–1284)</td>
</tr>
<tr>
<td>Pil-1-6</td>
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<td>CAAS GAGNNRKLI WGLGTSLAVNPN</td>
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<tr>
<td>Pil-4-5</td>
<td>ADV21S1A1N</td>
<td>CAAS GAGNNRKLI WGLGTSLAVNPN</td>
<td>TT(1272–1284)</td>
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**Table 2. TCR sequence analysis of TT-specific T cell clones**

<table>
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<tr>
<th>T cell clone</th>
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<th>TCR V\textsubscript{\textbeta} junctional sequence</th>
<th>Peptide specificity</th>
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<tr>
<td>Pil-1-2/33</td>
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</table>

**Alteration of DRB1*0301 (DR17) TT ligands at position P8 influences T cell activation**

To explore the proposed interaction between the arginine of the CDR3\textbeta loops and the corresponding aspartate at P8 of the TT peptide core in more detail, peptide analogues D\rightarrow E, D\rightarrow L and D\rightarrow K at P8 in these TT peptides were synthesized (see Table 1). These single amino acid alterations did not interfere with the binding capacity of the TT peptide analogues to DRB1*0301 (DR17) molecules (Table 1). Alterations at relative position P8 had little effect in TT(279–296) variants and even improved binding to DR17 in TT(1272–1284) variants. As shown in Fig. 5, TT(1272–1284)-specific T cell clones Pil-1, Pil-36, Pil-4 and Pil-6 were highly responsive to the wild-type peptide as well as to the previously described shorter variant TT(1273–1284) (20), whereas the exchange of the negatively charged residue aspartate at position P8 of the TT(1272–1284) sequence reduced T cell proliferation activity in a dose-dependent manner. As expected, the most prominent effect was observed by introducing an inversely charged residue (lysine for aspartate) which further reduced the T cell response activity of at least one order of magnitude. When the responses of T cell clone Pil-45 to the TT(279–296) peptide variants were tested, a similar, although not identical pattern was observed. As expected, TT(279–296) analogues showed a stepwise reduction of proliferation depending on the charge of peptide residue at P8 (TT290, Fig. 3). Wild-type TT(279–296) elicited the strongest proliferation, followed by TT(D290L) and TT(D290K) variants respectively. Surprisingly, the conservative alteration at P8 from aspartate to glutamate at TT290 almost abrogated the T cell response. It seems likely that the decreasing response to TT(D290E) variant peptide of clone Pil-45 is due to the increased size of glutamate versus aspartate.

Furthermore, we examined whether the described altera-
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Fig. 5. Alteration at the relative position P8 affects T cell proliferation of TT(1272–1284)-specific clones depending on the charge and size of the altered residue. T cell clones were stimulated with the wild-type peptide TT(1272–1284) (12), and the P8 variants TT1282D→L, D→K and D→E.

...tions at P8 had an influence on the pattern of the cytokine production of the T cells, possibly by creating altered peptide ligands (33,34). Secretion of IFN-γ, IL-10 and IL-4 was indistinguishable in the original peptide and the P8 analogues (data not shown). These data clearly confirm the hypothesis of a strong interaction between the aspartate at P8 of the peptide core and the N-terminal arginine of the TCR CDR3β loop in five different receptor–ligand pairs. We demonstrate the critical role of this ‘fixation point’ especially in the case of the major immunodominant TT-derived peptide TT(1272–1284) which results in the recruitment of a broader T cell repertoire specific for this epitope.

Discussion

We describe in this study structural features of TCR–MHC class II–peptide interactions with the aim to better understand why the T cell response is directed against few prominent epitopes, a phenomenon which is called immunodominance. We choose the TT as a well-characterized model antigen (19,20,35,36).

Several aspects considered to be involved in immunodominance (37) are investigated.

Antigen processing of antigens—predetermination of immunodominance

As a fundamental prerequisite of the T cell response, internalized protein antigens must be proteolytically cleaved to give rise to the epitopes that will bind to MHC class II. Previous in vitro processing studies of TT (36) confirmed that the TT-derived epitope TT(1061–1075) (described here for the first time) and TT(1272–1284) were not destroyed after incubation of the TT C-fragment with asparaginyl endopeptidase. Remarkably, Hewitt et al. demonstrated that the TT(1272–1284) epitope was flanked by cathepsin E and D cleavage sites (35). Since the sequence of the epitope TT(279–296) is not included in the TT C-fragment, there were no data available on the cleavage sites of asparaginyl endopeptidase, cathepsin E or D in this region. Furthermore,
only one short peptide [TT(951–954)] was recovered from the N-terminal half of the TT C-fragment (residues 865-1114) which may represent the inability of both cathepsins to cleave this part of the molecule (35). Despite the lack of information about the proteolytic cleavage sites around the T cell epitopes TT(279–296) and TT(1061–1075), we demonstrated that all three epitopes are generated during the processing in autologous APC and form T cell epitopes.

**Peptide competition for class II presentation?**

The second proposed requirement concerning immunodominance of a given peptide is the binding to the MHC molecule. Thus, if such peptides were generated from antigen degradation they should efficiently compete for MHC binding in the loading compartment (7,38). If this hypothesis is valid we should observe a good correlation between the immunogenicity of TT-derived epitopes and their binding capacity to purified DRB1*0301 molecules, but we were not able to confirm such a correlation in all cases. The strongest T cell response was found with TT(1272–1284) (Fig. 5 and data not shown), which binds only moderately to DRB1*0301 (Table 1). Thus, other mechanisms like peptide editing by HLA-DM may have an influence on the presentation of T cell epitopes. However, we could not examine whether DM sensitivity is different in TT(1272–1284) bound to DRB3*0101 or DRB1*0301. A recent study demonstrated cross-presentation of antigenic peptides by DR52a and DR17 molecules (23) since the binding motifs of both alleles were found to be almost identical (23,25). In this regard, we favor the possibility that TT(1273–1284) or TT(1272–1284) is able to bind to both MHC alleles and it primarily depends on the TCR structure which complex is preferentially recognized. Such a promiscuous binding of a given peptide would clearly broaden the repertoire of specific T cells and therefore contribute to immunodominance.

### A structural view on the T cell recognition of immunodominant epitopes

To investigate another parameter involved in immunodominance, we analyzed the physical interaction between the TCR and the TT-derived peptides. Based on the X-ray crystal structure data of the DRB1*0301–CLIP complex (9) we predicted the conformation of the bound peptides and potential TCR contact residues. The currently known conformations of MHC class II-bound peptides are remarkably similar (26,27). Since there is growing evidence that DRB3*0101 and DRB1*0301 binding peptides use identical or very similar anchor residues (23,25), we can assume that the potential TCR contact residues of TT(1272–1284) are identical if the peptide is bound to DRB3*0101 or DRB1*0301.

The sequence analysis of the TCR heterodimers recognizing the TT-derived peptides on the DRB3*0101/ DRB1*0301 background revealed two major characteristics of our studies. First, the immunodominant epitope TT(1272–1284) was capable of stimulating at least four different T cell clones (in the context of the autologous DRB3*0101/ DRB1*0301 MHC restriction) derived from the same donor. Second, TT(279–296) and TT(1272–1284), which share an asparagine at the TCR contact site P8, were recognized by TCR with an arginine at the third position of the CDR3β loop, suggesting a potential salt bridge between these corresponding residues. Further support for this idea was the observation that T cell clones Pii-2 and Pii-33 were not able to respond to TT(1272–1284) despite the fact that they express the same TCR α chain as the TT(1272–1284)-specific clone Pii-4 but differ in their CDR3α sequence (Table 2).

The phenomenon of different TCR recognizing the same MHC–antigen complex is known as synonymous TCR (39) and it is based on the determination of X-ray crystal structures of two TCR–MHC–peptide complexes where two different TCR (derived from T cell clones of two individuals) recognized the same MHC–peptide complex (40). Additionally, it was recently shown that the recognition of a HTLV-I Tax peptide bound to HLA-A2 by two human TCR is also possible if both T cell clones differ completely in their fine specificity for all contacting peptide residues (41). In the case of the HTLV-I Tax peptide, the stimulation of different T cell clones by a single MHC–peptide complex is facilitated by a high degree of degeneracy of the TCR. Since we raised four synonymous TT(1272–1284)-specific T cell clones by re-stimulating with TT, this high frequency of clones derived from a single donor may reflect the high potency of this dominant epitope to stimulate T cells. Furthermore we observed a similar dominant T cell response to TT(1272–1284) among a series of other DRB1*0301-positive donors (data not shown) compared to other less prominent T cell epitopes of TT. Previously described T cell responses against TT-derived peptides with the same nonamer core, i.e. TT(1273–1284) (20) and TT(1274–1284) (24), also support the importance of this immunodominant epitope within the panel of TT-derived antigenic peptides.

We confirmed the strong interaction between aspartate at P8 of TT(279–296) and TT(1272–1284) and arginine of the TCR CDR3β loop by single amino acid alterations of the antigenic peptides. The hierarchy of alterations at P8 paralleled closely the expected degree of T cell response regarding the postulated ionic interaction. Thus, strong reduction or even complete abrogation of T cell response was achieved by inversion of the charge in residue at P8. Two surprising exceptions were obtained after stimulation with the conservative exchange of aspartate to glutamate at P8. The TT(1272–1284)-specific T cell clone Pii-4 responded significantly stronger to glutamate at P8, whereas the TT(279–296)-specific clone Pii-4S failed to recognize the modified peptide. Interestingly, the TT(1272–1284)-specific clones Pii-4 and Pii-36 use the same TCR sequences with the exception of the CDR3α and CDR3β loops. Regarding the CDR3 sequences, one possible explanation could be the variable length of the CDR3β loops of both T cell clones. It has been reported previously (4,32,42,43) that CDR3β may interact with the peptide segment P5 to P8. Such a simultaneous interaction between the CDR3β loop and the antigenic peptide residues P5 and P8 is possible either if the orientation of the TCR–class II interaction is orthogonal as recently reported (44) or if it is diagonal as shown previously in the TCR–pMHC class I crystals (4,40,42). In this more recent study (44), the crystal structure analysis of the scDC (mouse TCR–CAI/IAβ) complex showed that the CDR3β loop interacts only with the P8 glutamine side chain of conalbumin (CA) peptide. Detailed analysis of this crystal structure revealed that it is probable that CDR3β residues could interact with P5 and
P8 simultaneously in other TCR–pMHC class II complexes (44). This suggests that the smaller loop of clone Pil-4 may prefer the larger residue volume of glutamate at the relative position P8, thus enabling the smaller CDR3β loop to contact the antigen residues P5 and P8 simultaneously, whereas aspartate at P8 fails to interact with arginine in CDR3β to the same extent as with glutamate at P8. In contrast to the other TT(1272–1284)-responding T cell clones Pil-1, Pil-6 and Pil-36, the strong response of Pil-4 to the P8 D–E variant may also reflect different peptides involved in positive selection during T cell development in the thymus. On the other hand, the abrogated response of Pil-45 against the D–E variant of TT(279–296) is probably caused by steric hindrance due to the increased bulkiness of the residue at P8.

Taken together, our data show that a particular strong interaction between a TCR contact site of the antigenic peptide and a corresponding residue of the CDR3β segment of the TCR may contribute to immunodominance. Such strong contacts could act as a ‘fixation point’ which facilitates the recognition of a prominent epitope peptide by a broader repertoire of specific T cells expressing synonymous TCR.

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Abbreviations

APC antigen-presenting cell
CA conalbumin
CDR complementarily-determining region
PBMC peripheral blood mononuclear cell
TT tetanus toxin

References

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