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*J Immunol* (2004) 173 (9): 5610–5616.

<https://doi.org/10.4049/jimmunol.173.9.5610>

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# Conformational Restraints and Flexibility of 14-Meric Peptides in Complex with HLA-B\*3501<sup>1</sup>

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Human HLA-B\*3501 binds an antigenic peptide of 14-aa length derived from an alternative reading frame of M-CSF with high affinity. Due to its extraordinary length, the exact HLA binding mode was unpredictable. The crystal structure of HLA-B\*3501 at 1.5 Å shows that the N and C termini of the peptide are embedded in the A and F pockets, respectively, similar to a peptide of normal length. The central part of the 14-meric peptide bulges flexibly out of the groove. Two variants of the alternative reading frame of M-CSF peptide substituted at P2 or P2 and P9 with Ala display weak or no T cell activation. Their structure differs mainly in flexibility and conformation from the agonistic peptide. Moreover, the variants induce subtle changes of MHC  $\alpha$ -helical regions implicated as critical for TCR contact. The TCR specifically recognizing this peptide/MHC complex exhibits CDR3 length within the normal range, suggesting major conformational adaptations of this receptor upon peptide/MHC binding. Thus, the potential antigenic repertoire recognizable by CTLs is larger than currently thought. *The Journal of Immunology*, 2004, 173: 5610–5616.

Cytotoxic CD8<sup>+</sup> T cells recognize small antigenic peptides derived from endogenous proteins bound to MHC class I molecules. Such epitopes are normally 8–10 aa in length and are mainly generated by proteasomal degradation. Defective translational products were suggested as the main source (1, 2). Besides these conventional epitopes, originating from nominal proteins, a whole set of epitopes has been identified that is produced from unconventional sources. These include posttranslational modified proteins, spliced peptides, translational products of retained introns, and alternative translational reading frames (3–6). A prototypic example of the latter was discovered when a tumor-associated Ag, recognized by a tumor-infiltrating CD8<sup>+</sup> T cell clone was characterized. This CTL clone recognized an antigenic peptide presented by HLA-B\*3501 that was encoded by an alternative reading frame of the human M-CSF (alt.M-CSF)<sup>4</sup> and displayed the extraordinary length of 14 aa (7).

Based on the observation that a nonameric peptide already had a tendency to bulge out of the groove to fit (8), it was suggested that longer peptides would bind more weakly to HLA-B\*3501. In contrast, the alt.M-CSF peptide exhibited binding affinity to HLA-B\*3501 similar to a nonamer T cell epitope derived from a mutated caspase 8 (CASP8), which was considered to be a high-affinity binding peptide (9, 10). Half-maximal concentration required to inhibit binding of a reference peptide (IC<sub>50</sub>) to HLA-B\*3501 was <5  $\mu$ M (7, 9). Furthermore, binding studies using peptide analogs suggested that the alt.M-CSF peptide is bound via its two extremities, and therefore the remaining central part was expected to bulge out of the groove to compensate for length (7).

Therefore, we determined the crystal structures of HLA-B\*3501 in complex with the 14-mer alt.M-CSF peptide. The CASP8 peptide exhibiting similar binding affinity was resolved in complex with HLA-B\*3501 for comparison. To obtain insights into the structural differences of alt.M-CSF peptide variants with altered biological activity, we selected two alanine-substituted variants with nearly identical binding affinity to HLA-B\*3501. Whereas the alanine substitution at P2 of the alt.M-CSF peptide rendered the resulting variant (P2Ala) to a weak agonist, the additional alanine substitution at P9 (P2AlaP9Ala) totally abrogated the weak T cell response induced by the P2Ala peptide (7). The P2Ala variant was selected because of the conservative amino acid exchange at a position normally not in contact with the TCR (11, 12) and because of its characterization as potential HLA-B\*3501 anchor amino acid (13).

The overall structures of the alt.M-CSF/HLA-B\*3501 complexes reveal that these 14-mer peptides are mainly bound via their N- and C-terminal 3 and 2 aa, respectively, and bulge out with their remaining central parts. The flexibility and conformation of the agonistic peptide differ from the variants mainly in this central part. In addition, the variants induce subtle shifts in regions of the  $\alpha$ -1 and  $\alpha$ -2 helices of HLA-B\*3501 that were shown to be critical for TCR contact in previously investigated HLA class I molecules (14–16).

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Received for publication April 19, 2004. Accepted for publication August 17, 2004.

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<sup>1</sup> M.P.-K. is supported by the VolkswagenStiftung and Deutsche Forschungsgemeinschaft. S.W. is supported in part by Deutsche Forschungsgemeinschaft and Deutsche Krebshilfe.

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<sup>4</sup> Abbreviations used in this paper: alt.M-CSF, alternative reading frame of M-CSF; CASP8, caspase 8; r.m.s.d., root mean squared difference; Ebna, EBV nuclear Ag; P2Ala and P2AlaP9Ala, variants of alt.M-CSF peptide residue 4–17 LPAVVGLSPGEQY with Ala in P2 and P2, P9, respectively.

Table I. Data processing statistics

	HLA-B*3501 in Complex with			
	alt.M-CSF	P2Ala	P2AlaP9Ala	CASP8
Resolution (Å)	19.0–1.48	21.0–1.48	22.0–1.48	30.0–2.3
Wavelength (Å)	1.05	1.05	1.05	1.5418
Space group	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>
Lattice constants				
<i>a</i> (Å)	50.828	50.771	50.827	50.608
<i>b</i> (Å)	81.663	81.209	81.749	81.371
<i>c</i> (Å)	109.798	110.424	110.812	109.482
Unique reflections	72270	70965	72765	19977
Redundancy	3.4	4.8	3.8	3.4
Completeness (%)	93.7 (92.0)	91.7 (79.8)	93.4 (85.3)	96.8 (98.9)
<i>R</i> <sub>meas</sub> (%) <sup>a</sup>	6.1 (45.2)	11.5 (30.8)	10.9 (43.9)	7.3 (23.6)
$\langle I \rangle / \langle \sigma \rangle$	5.2 (1.6)	3.8 (1.3)	3.5 (2.3)	6.2 (0.8)
Wilson plot B (Å <sup>2</sup> )	18.2	14.8	19.2	32.0

<sup>a</sup> *R*<sub>meas</sub> was calculated with SCALA (44); values in parentheses are for the highest resolution shell. meas, Measured.

## Materials and Methods

### Expression, purification, and crystallization of HLA-B\*3501 complexes

HLA-B\*3501 (amino acid residues 1–277) and  $\beta_2$ -microglobulin (kindly provided by Prof. Dr. A. Ziegler, Charité, Berlin, Germany) were expressed in *Escherichia coli*, purified, and refolded with synthetic HPLC-purified peptides (kindly provided by Dr. W. Tegge, German Research Centre for Biotechnology (GBF)) as described (8). The refolded peptide/MHC complexes were purified by gel filtration chromatography using a Superdex 75 HR column (Pharmacia Biotech, Uppsala, Sweden) and concentrated further using Vivaspin concentrator units (Vivascience, Hannover, Germany) in 20 mM Tris-HCl (pH 7.5) and 150 mM NaCl. Protein concentrations of peptide/MHC complexes exceeding 50 mg/ml by OD (Eppendorf BioPhotometer; Eppendorf, Hamburg, Germany) reproducibly crystallized using sitting drop vapor diffusion at 19°C by mixing in a 1:1 ratio of protein to crystal solution (20% (v/v) polyethylene glycol 4000).

### Data collection and processing

Before diffraction experiments, crystals were washed in reservoir solution with 20% glycerol and flash-cooled in liquid nitrogen. Data of the 14-mer/HLA-B\*3501 complexes were collected at the Max Planck Gesellschaft-GBF beamline BW6 at the Deutsches Elektronen Synchrotron synchrotron

at a wavelength of 1.05 Å with a Marresearch (Norderstedt, Germany) charge-coupled device detector. The CASP8/HLA-B\*3501 complex data were collected at a Rigaku (Tokyo, Japan) rotating anode generator, operated at 50 kV and 90 mA, with Cu K radiation using a Raxis IV++ (Rigaku, Sendagaya, Japan) detector. Crystals were cooled by a nitrogen gas stream to 100 K. Data were processed using the programs Mosflm and Scala of the CCP4 program suite. Crystal data are summarized in Table I.

### Molecular replacement and model refinement

The initial structure solution proceeded by molecular replacement using the coordinates of HLA-B\*3501 (Brookhaven Protein Data Bank (PDB) no. 1A1N) (17), stripped of the bound peptide, and the Molrep program (CCP4), which showed a clear peak of 8.5  $\sigma$  in the rotation function, 47  $\sigma$  with an *R* factor of 36.2, and a correlation factor of 0.679 in the translation function. The peptide was traced in electron density, and difference maps were calculated with Refmac (CCP4). The structures were refined using O (18) and Refmac (CCP4), in the last stages with TLS parameters. Water molecules were added using Arp/Refmac (CCP4) until there was no further decrease in the free *R* factor. The final refinement statistics are summarized in Table II. Coordinates for all crystal forms of HLA-B\*3501 will be deposited in the PDB.

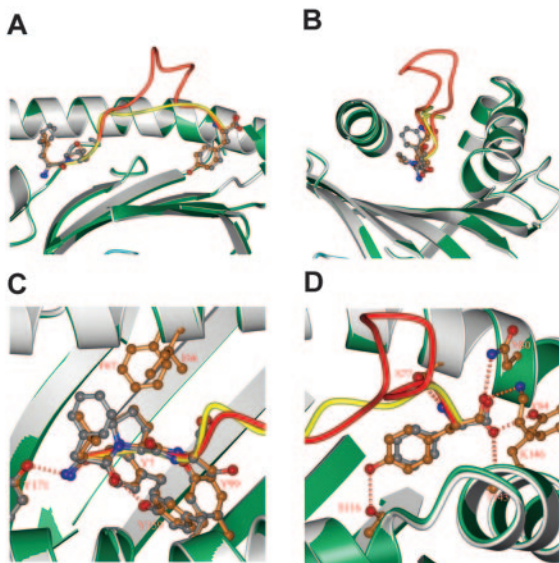
Table II. Refinement statistics

	HLA-B*3501 in Complex with			
	alt.M-CSF	P2Ala	P2AlaP9Ala	CASP8
Resolution range (Å)	19.0–1.48	21.0–1.48	22.0–1.48	30.0–2.3
<i>R</i> <sub>work</sub> (%) <sup>a</sup>	18.3 (27.3)	18.8 (29.0)	18.4 (22.1)	17.1 (19.0)
<i>R</i> <sub>free</sub> (%) <sup>a,b</sup>	21.8 (28.1)	22.8 (31.1)	21.6 (27.1)	25.1 (31.5)
No. reflections	68,581	70,252	69,053	18,965
No. atoms	3,758	3,760	3,726	3,348
No. waters	572	576	544	182
Mean B (Å <sup>2</sup> )	12.9	10.8	13.7	28.7
Ramachandran plot				
Favored (%)	92.3	90.9	92.9	90.7
Allowed (%)	6.8	8.6	6.5	8.7
Generous (%)	0.9	0.6	0.6	0.6
Disallowed (%)	0.0	0.0	0.0	0.0
Coordinate error <sup>a,c</sup> (Å)	0.054	0.057	0.047	0.158
r.m.s.d. from ideality <sup>a</sup>				
Bonds (Å)	0.019	0.020	0.014	0.040
Angles (°)	1.7	1.8	1.5	2.6
Dihedrals (°)	6.7	6.2	6.4	7.6
Chiral centers (Å <sup>3</sup> )	0.112	0.118	0.094	0.168

<sup>a</sup> Calculated with REFMAC5 (44, 46).

<sup>b</sup> *R*<sub>free</sub> data set consisted of 5% of the unique reflections Ramachandran plot calculated with PROCHECK (44, 45).

<sup>c</sup> Based on maximum likelihood.



**FIGURE 1.** Superimposition of the 14-mer alt.M-CSF and 9-mer CASP8 peptides in the HLA-B\*3501 groove. The alt.M-CSF (red) and CASP8 (yellow) peptides are anchored similarly at their N and C termini, but the central part of the alt.M-CSF peptide protrudes extraordinarily out of the groove. Only the N- and C-terminal amino acids are presented as ball-and-stick (brown, alt.M-CSF; dark gray, CASP8). *A*, Side view of the peptide/MHC complex. The MHC  $\alpha$ -2 helix has been clipped to provide better visibility of the peptides; the  $\alpha$ -1 helix is in the background (green, alt.M-CSF; gray, CASP8). *B*, In the orthogonal view, the  $\alpha$ -1 helix is present on the *left*, and the  $\alpha$ -2 helix on the *right*. *C* and *D*, The alt.M-CSF and the CASP8 peptides show similar side-chain interactions with the MHC at their N and C termini and are similarly bound in the A (*C*) and F pockets (*D*) of HLA-B\*3501, respectively. Only the C-terminal interaction of the CASP8 peptide to S116 of the MHC is lost because of a C-terminal phenylalanine instead of tyrosine (*D*). Hydrogen bonds are indicated as red broken lines.

#### Cloning and sequencing of TCR $\alpha\beta$ of CTL clone 403A/9

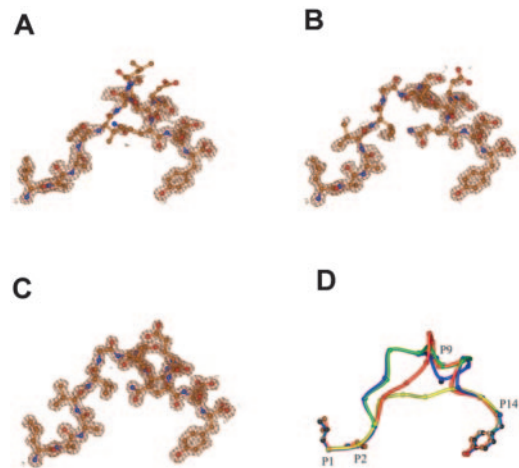
Total RNA was isolated from  $1 \times 10^5$  cells of CTL 403A/9 using a total RNA isolation kit (Qiagen, Hilden, Germany). cDNA was synthesized using oligo(dT) primers and murine leukemia virus reverse transcription polymerase (Invitrogen Life Technologies, Carlsbad, CA) following the manufacturer's recommendations. CDR3 sequences of TCR $\alpha$  and  $\beta$  chains were amplified using high-fidelity Pfu polymerase (Promega, Madison, WI). Primers were as follows: for the TCR $\alpha$ , KWA-1, 5'-CAT ATG GAC CAG GTG ACG CAG AGT C-3', and KWA-2, 5'-CTC GAG GGA ACT TTC TGG GCT GGG GA-3'; and for the TCR $\beta$ , KWA-4, 5'-CAT ATG GGA GTC ACC CAA AGT CCC ACA-3', and KWA-5, 5'-CTC GAG AGC GCC GAG GCC TGG GGT AGA-3'. *Nde*I and *Xho*I sites (underlined) were introduced for intended expression cloning. PCR products were cloned into pCR4.1 (Invitrogen Life Technologies) and analyzed by automatic DNA sequencing (GATC Biotech, Konstanz, Germany).

## Results

#### Structure of HLA-B\*3501 in complex with a 14-mer alt.M-CSF peptide

The crystal structure of the human MHC class I molecule HLA-B\*3501 in complex with a 14-aa long peptide derived from alt.M-CSF (residues 4–17 of alt.M-CSF: LPAVVGLSPGEQEY) was determined at a resolution of 1.5 Å. For comparison, HLA-B\*3501 in complex with a 9-mer peptide derived from a mutated CASP8 (residues 476–484: FPSDSWCYF; Ref. 10) was included in this study.

The overall structure of HLA-B\*3501 in complex with the alt.M-CSF peptide shows standard MHC folds and does not differ significantly from the previously described HLA-B\*3501 struc-



**FIGURE 2.** Flexibility and conformation of the different 14-mer alt.M-CSF peptides. Alt.M-CSF agonist (*A*), P2Ala (*B*), and P2AlaP9Ala (*C*) peptides that exhibit similar binding affinities to HLA-B\*3501 ( $IC_{50}$ : 3.1, 2.6, and 1.7  $\mu$ M, respectively), show increasingly defined electron density maps, indicated by the contoured area around the ball-and-stick presentation of the peptides. This suggests decreasing flexibility in the central part of these peptides in order from *A* to *C*. *D*, Superposition of the alt.M-CSF agonist (red), P2Ala (green), and P2AlaP9Ala (blue) as tube representation illustrates the diverging traces of the peptides. The CASP8 trace (yellow) is included for comparison. The conformation and flexibility of the alt.M-CSF peptide has been confirmed in an independent crystal resolved at 1.8 Å (not shown).

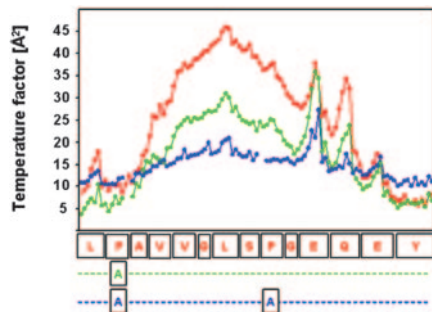
tures in complex with an octamer Nef peptide at 2.0-Å resolution (PDB no. 1A1N) (17) or a nonamer EBV nuclear Ag (Ebna)3C peptide at 2.5-Å resolution (PDB no. 1A9E) (8). The corresponding maximum root mean squared difference (r.m.s.d.) for  $C\alpha$  atoms of HLA-B\*3501 is between 0.4 and 0.7 Å compared with the Nef/HLA-B\*3501 and Ebna3C/HLA-B\*3501 complexes, respectively.

The exceptionally long 14-mer alt.M-CSF peptide is anchored to HLA-B\*3501 like the CASP8 peptide via its N-terminal 3 aa and C-terminal 2 aa, respectively (Fig. 1). Differences between corresponding  $C\alpha$  atoms in the N- and C-terminal positions between the alt.M-CSF and the CASP8 peptide are only in the range of 0.3–0.5 Å. The conformation of the alt.M-CSF peptide remains close to that of the CASP8 peptide up to P4 and is nearly identical at P13/P14 with the corresponding P8/P9 residues of CASP8. Severe differences between the two peptides are obvious from P5 to P11 of the alt.M-CSF peptide compared with P5 to P6 of the CASP8 peptide. Whereas the CASP8 peptide remains within the binding groove, thus forming a plain surface with only a few side chains exposed to the TCR, the central part of the alt.M-CSF peptide is protruding like a loop from the plain formed by the two  $\alpha$  helices of the groove (Fig. 1, *A* and *B*). This provides considerably more than a few side chains for interaction with the TCR.

#### Structure of HLA-B\*3501 with alt.M-CSF peptide variants displaying altered biological activity

Two peptide variants of alt.M-CSF in complex with HLA-B\*3501 were determined at 1.5-Å resolution, in addition. The alanine substitution at the anchoring residue P2 results in a weakened T cell activation (7), whereas the additional alanine substitution at P9 totally abrogates T cell recognition. Both variant peptides differ from the agonist mainly in terms of flexibility and conformation. Fig. 2*A* shows that the electron density of the agonist alt.M-CSF peptide is well defined only at its first 4 aa and C-terminal 6 aa, whereas the electron density is broken between residues P4 to P9





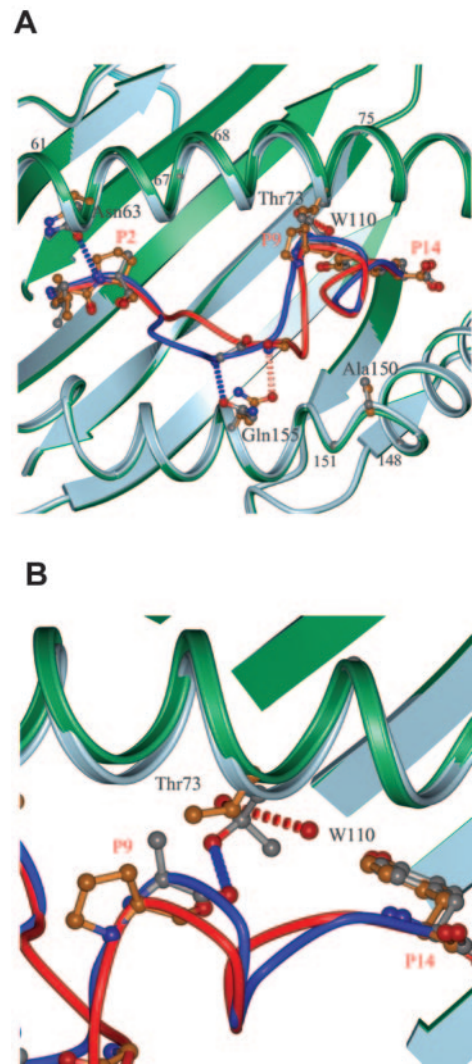
**FIGURE 3.** Difference in temperature factors of the 14-mer alt.M-CSF peptides. Differences in flexibility of the central parts of the alt.M-CSF (red), P2Ala (green), and P2AlaP9Ala (blue) peptides are visible in the temperature factor plot. Dots indicate single atoms of the peptide. The number of dots corresponding to individual amino acids is indicated by the width of each box below the graph (single-letter code). Gaps were introduced to accommodate the different number of atoms of the substituted amino acids. The corresponding temperature factor values are presented on the y-axis in square angstroms.

at several positions, indicating flexibility in that particular part of the peptide. In contrast, both variants show a more defined electron density in this region, where the P2Ala is better defined as the agonist peptide (Fig. 2B), and the P2AlaP9Ala is completely defined (C).

The differences in flexibility of the three 14-mer alt.M-CSF peptides are corroborated by temperature factor determinations. As shown in Fig. 3, the N- and C-terminal parts of the three peptides embedded in the A and F pockets, respectively, do not have conformational freedom, indicated by the low temperature factor. In contrast, the central parts bulging out of the groove show an increased temperature factor. Correspondingly, the agonistic peptide shows the highest temperature factor, whereas weaker or no T cell recognition of the variant peptides correlates with gradually decreasing temperature factor, i.e., lower flexibility. These differences in flexibility are not caused by crystal contacts, because the neighboring molecules to the bulging part of the peptides are  $>8$  Å apart.

Concerning the conformation of the different 14-mer peptides, the proline-to-alanine substitution of the anchoring P2 does lead to changes in the  $C\alpha$  trace of the variant peptides compared with the agonist (Fig. 2D). The  $\phi/\psi$  angles of the N-terminal 3 aa of the variants differ up to  $30^\circ$  due to the alanine substitution. Thus, an additional hydrogen bond of the amide group of alanine at P2 to Asn<sup>63</sup> of the HLA molecule is formed (Fig. 4). Consequently, the  $C\alpha$  at P2 differ by 0.5 Å, whereas the  $C\alpha$  of P3 remains virtually identical for all three 14-mer peptides. This is followed by a divergence from the  $C\alpha$  traces of the agonist and CASP8 peptides at P4 (Fig. 2D). From there, both variants follow a similar trace up to P7 (Fig. 2D) and form a strong hydrogen bond between the backbone nitrogen of glycine at P6 and the Gln<sup>155</sup> side chain of the HLA molecule (Fig. 4). This contributes to the reduced flexibility and improved electron density of these peptides. The agonistic peptide, due to its different trace, forms a weak hydrogen bond at this position. This might explain the residual electron density of the agonistic peptide at this position (Fig. 2A).

From P9 to P12, the three peptides show large positional differences. The alanine substitution in P9 of the P2AlaP9Ala results in a difference of 0.5 Å from the P2Ala variant peptide, and places the  $C\alpha$  of this amino acid 1.6 Å apart from the original agonistic epitope. This correlates with a different side chain rotamer of Thr<sup>73</sup> in the P2AlaP9Ala/HLA-B\*3501 complex, allowing an additional hydrogen bond to the alanine at P9 of the peptide (Fig. 4). From P9



**FIGURE 4.** HLA-B\*3501  $\alpha$ -helical changes induced by the alanine-substituted alt.M-CSF variants. Only the agonistic alt.M-CSF peptide (red) and P2AlaP9Ala (blue) are displayed. *A*, Compared with the agonistic peptide, the alanine exchange at P2 of the P2Ala (not shown for clarity) and P2AlaP9Ala peptides results in an additional hydrogen bond to Asn<sup>63</sup> of the HLA molecule indicated by the broken blue line. This leads to a deviation of the  $\alpha$ -1 helix (gray) toward the binding groove. Both variants also form a strong hydrogen bond at Gln<sup>155</sup> (broken blue line), explaining their reduced flexibility and improved electron density. In contrast, the agonistic peptide shows only a weak hydrogen bond (red broken line) to the flipped Gln<sup>155</sup>. The alanine substitution at P9 of the P2AlaP9Ala peptide enables a unique hydrogen bond to Thr<sup>73</sup> of the  $\alpha$ -1 helix. This leads to the extrusion of a water molecule (W110) and a deviation of the  $\alpha$ -1 helix of 1.1 Å toward the binding groove compared with the agonistic and the P2Ala (not shown) peptide as presented in *B* at a higher magnification and slightly angulated perspective. This results also in a deviation of the  $\alpha$ -2 helix around residue Ala<sup>150</sup> (from Glu<sup>148</sup> to Arg<sup>151</sup>) of 0.5 Å on the opposite side of the groove, indicated in *A*. Hydrogen bonds are colored similar to the corresponding peptide.

on, the P2Ala peptide joins the trace of the agonistic peptide. The P2AlaP9Ala peptide, in spite of a close similarity at P10 and P11, deviates by 1 Å at P12 from its two counterparts. This deviation gradually decreases but is still visible at the C-terminal residue P14 with a difference of 0.4 Å. Independent of these differences, all three alt.M-CSF peptides show a helical conformation between P9 and P12.

Table III. CDR3 regions of the alt.M-CSF-specific CTL clone 403A/9

CTL	V	CDR3 (Length)	C	Specificity
<b>TCRA<sup>a</sup></b>				
403A/9 <sup>b</sup>	CAV	QAGSNYQLIWGAGTKLI IKP (20)	DIQ	alt.M-CSF/B*3501
121	CAV	DITGNQFYFGTGTSLTVIP (19)	NIQ	CASP8/B*3501
A6	CAV	TTDSWGKLGQFGAGTQVVVTP (20)	DIQ	Tax/HLA-A2
JM22	CAG	AGSQGNLIFGKGTKLSVKP (19)	NIQ	MP/HLA-A2
L13	CIL	PLAGGTSYGKLTFGQGTILTVHP (22)	NIQ	Ebna3A/HLA-B8
<b>TCRB<sup>a</sup></b>				
403A/9 <sup>b</sup>	CAI	DTEISGANVLTFGAGSRLTVL (21)	EDL	alt.M-CSF/B*3501
121	CAS	SFSGGTGNTIYFGECSWLTVV (20)	EDL	CASP8/B*3501
A6	CAS	RPGLAGGRPEQYFGPGTRLTVT (22)	EDL	Tax/HLA-A2
JM22	CAS	SRSSYEQYFGPGTRLTVT (18)	EDL	MP/HLA-A2
L13	CAS	SLGQAYEQYFGPGTRLTVT (19)	EDL	Ebna3A/HLA-B8

<sup>a</sup> TCR sequences from the following references: CTL 121 (9), A6 (35), JM22 (16), L13 (47).

<sup>b</sup> TCRAV20S1; TRAJ33\*01 (GenBank accession no. AY513882); TCRBV5S6, TCRD1\*01, TCRBJ2-6\*01, TCRBC2\*02 (GenBank accession no. AY513883).

The resolution at 1.5 Å allowed a clear assignment of the water molecules being part of the different 14-mer peptide/MHC complexes. Such water molecules act as space-filling molecules between the MHC binding groove and the peptide part bulging out. Corresponding to the different conformations of the 14-mer alt.M-CSF peptides, the positions and number of the water molecules beneath the bulgy part of the peptide deviate between the three complexes to various degrees. For instance, the proline-to-alanine substitution at P9 besides the additional hydrogen bond to the Thr<sup>73</sup> side chain of the HLA molecule, leads to a displacement of a water molecule. This water molecule is hydrogen bonded to the Thr<sup>73</sup> side chain in the complexes with the P2Ala and agonistic peptide (Fig. 4).

#### Structural changes of HLA-B\*3501 caused by the alt.M-CSF peptide variants

The three HLA-B\*3501/14-mer peptide complexes not only differ in flexibility and conformation of the peptide but also exhibit several differences in the  $\alpha$  helices of the MHC class I molecule. The regions concerned have been implicated as critical for TCR contact (14–16). The proline-to-alanine exchange at P2 of the two variant peptides results in a nearly identical shift of 0.5 Å toward the binding groove in the  $\alpha$ -1 helix of HLA-B\*3501 around residue Asn<sup>63</sup> (from Asp<sup>61</sup> to Phe<sup>67</sup>) (Fig. 4). The corresponding r.m.s.d. value between both variants of less than 0.2 Å is within the range expected for regions being identical at the achieved resolution.

Additional changes are caused by the proline-to-alanine substitution at P9 of the P2AlaP9Ala peptide. First, around Thr<sup>73</sup> (forming the additional hydrogen bond with the P2AlaP9Ala peptide), the  $\alpha$ -1 helix deviates from Lys<sup>68</sup> to Arg<sup>75</sup> toward the binding groove with an r.m.s.d. value of 1.1 Å compared with HLA-B\*3501 in complex with the agonistic and the P2Ala peptide (Fig. 4). Second, in the  $\alpha$ -2 helix on the opposite side of the binding groove around residue Ala<sup>150</sup> (from Glu<sup>148</sup> to Arg<sup>151</sup>), a deviation with an r.m.s.d. value of 0.5 Å is observed (Fig. 4).

#### The sequence of the TCR $\alpha\beta$ recognizing the 14-mer alt.M-CSF peptide bound to HLA-B\*3501

The CTL clone 403A/9 specifically recognizes the agonistic alt.M-CSF peptide presented by HLA-B\*3501. The TCR genes of this CTL were sequenced, and unique sequences were obtained for the  $\alpha$ - and  $\beta$ -chain. CDR3 of both chains are displayed in Table III in comparison with the TCR specific for the CASP8 peptide presented by HLA-B\*3501 (10). The CDR3s of alt.M-CSF-specific TCR $\alpha$  and  $\beta$  chains are 1 and 2 aa longer, respectively, and are

within the range of CDR3s of TCR recognizing standard length peptides, e.g., the structurally resolved HLA-A2-restricted TCR A6 (19) and JM22 (20), or the HLA-B8-restricted TCR L13 (16) (Table III). Positioning of the structure of these TCR onto the antigenic surface of alt.M-CSF/HLA-B\*3501 complex led to steric collisions. However, because these TCR are adapted to normal length peptide/MHC complexes, a TCR recognizing bulgy peptides might display considerably more adaptive flexibility.

## Discussion

Antigenic peptides normally bind to MHC class I molecules via their N and C termini within conserved pockets (21). There are only two exceptions reported for classical MHC class I molecules: 1) an additional amino acid extends from either the peptide C terminus out of the F pocket (22) or the N terminus out of the A pocket (23); 2) the N terminus is shortened by one residue, therefore not reaching the A pocket (24). The crystal structure of HLA-B\*3501 in complex with the 14-mer alt.M-CSF peptide clearly shows that this antigenic peptide follows the general rules of MHC binding. Despite its extraordinary length, it is bound via its N- and C-terminal 3 and 2 aa, respectively, similar to the nonameric CASP8 and Ebna3C peptides (8). The central part of the alt.M-CSF peptide bulges flexibly out of the HLA groove to adapt for the excessive length. Although the protruding part shows only minor interactions with the MHC molecule, this obviously does not measurably weaken the affinity to the MHC as determined previously in a peptide competition assay compared with the 9-meric CASP8 peptide (7), a peptide that is embedded flat in the binding groove. Thus, high-affinity MHC binding as the minimum requirement for a potential T cell epitope is sufficiently warranted by only the N- and C-terminal anchoring as exemplified here for the alt.M-CSF peptide.

Bulging flexibly out of the groove to compensate for the excessive size has been observed previously for a collection of self-peptides of up to 11 aa in length in complex with human HLA-Aw68 (25). In addition, a variation of central flexibility of the peptide has been observed for the 13-mer MTF-E peptide bound to rat RT1-A<sup>a</sup> (26), which exhibits two different conformations, possibly associated with crystal-packing effects. These conformations have to be considered as the limiting conformations of a flexible peptide. The 13-mer MTF-E peptide has been characterized as rat minor histocompatibility Ag depending on an allelic form of rat TAP (27).

Our finding is of clinical relevance, because this antigenic 14-mer peptide is not predictable by current algorithms for HLA class

I-binding peptides because of its length (28). Peptide elution studies led to the identification of mainly 9-mer peptides bound to MHC class I (29, 30), representing the major pool of 80–90% of HLA class I-associated peptides (31) and T cell epitopes characterized so far (<http://syfpeithi.bmi-heidelberg.com>). However, longer peptides have to be considered as potential antigenic epitopes that can be recognized by T cells as shown in this study.

The alt.M-CSF peptide shows all of the features of a normal-length immunogenic peptide including the following: 1) standard embedding of the peptides' N- and C-terminal parts in the A and F pockets, respectively; 2) comparable MHC binding affinity correlated with nanomolar concentrations needed to induce half-maximal lysis of peptide-pulsed target cells (7); 3) TAP-dependent presentation (M. Probst-Kepper and B. J. van den Eynde, unpublished data); and 4) recognition by a normal TCR. The recent identification of an antigenic peptide of 12-aa length derived from the melanocyte differentiation Ag tyrosinase also presented by HLA-B\*3501 (32) corroborates our findings. Together, this opens the perspective that defects in Ag processing and presentation of tumor cells implicated by the presentation of longer peptides, e.g., due to TAP defects (33), not only represent a mechanism of tumor immune escape (34) but might also represent therapeutic options to use such longer peptides as potential targets for induction of a tumor-specific CD8 T cell response.

The prediction of such long peptides might be even further hampered. The possible variability of the amino acid sequence at position P2 seems greater than only proline, alanine, or serine, the most commonly observed amino acids at P2 in T cell epitopes presented by HLA-B\*3501 (<http://syfpeithi.bmi-heidelberg.com>), i.e., the 14-mer alt.M-CSF peptide could accommodate Met, Trp, and Gly at this position (7). It is unclear whether this observed variability is a particular feature of this protruding peptide only, or whether it could also be observed in naturally occurring peptides of normal or extended length.

Being fixed via its N and C termini and exhibiting only minor interactions with the MHC molecule in its central part, flexibility of the alt.M-CSF peptide is the consequence. This flexibility is impaired to different degrees in the variant peptides. Thus, it could be speculated that flexibility might be a prerequisite for proper T cell recognition of the alt.M-CSF peptide. In the variant peptides, the proline-to-alanine exchange at P2 results in an additional hydrogen bond to Asn<sup>63</sup> of the HLA molecule and a slight deviation of the  $\alpha$ -1 helix. The difference remote of P2 in the trace and flexibility of the variants seems to be related to the strong hydrogen bond of the glycine at P6 of the peptides to Gln<sup>155</sup>, explaining their reduced flexibility and improved electron density. The well-defined electron density, i.e., extreme rigidity, of the P2AlaP9Ala peptide might be further explained by the additional hydrogen bond between alanine at P9 of the peptide and Thr<sup>73</sup>, which is responsible for the deviation of the  $\alpha$ -1 helix at that position in the P2AlaP9Ala/HLA-B\*3501 complex and the corresponding  $\alpha$ -2 helix shift around Ala<sup>150</sup>. More important, the differences in the MHC  $\alpha$  helices are located in regions shown to be critical for TCR contact in other HLA class I molecules (14–16, 20, 35), with the P2AlaP9Ala introducing even more changes than the P2Ala peptide. It could be predicted that these subtle changes influence the proper energetic TCR footprint.

Thus, either the changes in flexibility and conformations of the alt.M-CSF peptide or the changes in the MHC  $\alpha$  helices induced by its alanine-substituted variants or both could influence the thermodynamics of TCR binding and TCR discrimination. In addition, the proline in P9 appears to be exposed to the TCR and might therefore be directly recognized by the TCR.

The CDR3 sequences of the  $\alpha$ - and  $\beta$ -chains of the TCR recognizing the alt.M-CSF peptide are within the size range of CDR3s of the TCR chains recognizing antigenic peptides of normal length. They do not display any special feature explaining a length adaptation of the TCR to the highly exposed residues of the peptide elevated above the binding groove. However, due to the exceptional length of the 14-mer peptide, the conformational adaptation might be limited and could possibly not adjust to a flat antigenic surface as the CASP8/HLA-B\*3501 complex. Therefore, even more dramatic structural reorganizations of the CDRs of the TCR than observed previously upon peptide/MHC binding (up to 15 Å) (11, 20, 36) might be expected.

The postulated flexibility for proper TCR recognition of the alt.M-CSF peptide extends the suggested dual-conformation hypothesis (37) based on distinct conformations observed with a nonamer peptide bound to HLA-B\*2705 (38) and the 13-mer MTF-E peptide bound to rat RT1-A<sup>a</sup> (26). For both cases, the conformational differences observed can be considered as the limiting conformations of a flexible peptide. But contrary to the observed peptide conformation in the unliganded peptide/MHC complexes, it can be expected that upon TCR binding the peptide adjusts to a unique conformation as observed with the peptide/MHC/TCR complexes analyzed so far (39).

Only the structural analysis of the TCR/alt.M-CSF peptide/MHC complex will allow a definite explanation. A nearly identical conformation of an agonistic and antagonistic Tax peptide bound to HLA-A2 appeared upon ligation with the specific A6 TCR (19). Nevertheless, large differences in binding free energy of the different TCR/peptide/MHC complexes were observed (40). Therefore, it will be interesting to visualize variations of the antigenic surfaces of the three alt.M-CSF peptides displaying different biological activities upon TCR binding.

It will also be interesting to address the question of TCR binding of the peptide/MHC complex with such long protruding peptides concerning the current models of TCR peptide/MHC interactions (the "two-step" and the "Tritope" model) (41, 42). The alt.M-CSF/HLA-B\*3501/TCR complex may elucidate some unexpected solutions to the structural requirements of TCR binding. The accepted diagonal mode of TCR recognition of MHC class I has recently been challenged by the finding of Buslepp et al. (43) describing that a TCR is able to recognize a peptide/MHC complex in an almost orthogonal mode. This has important consequences on the understanding of the selection of the TCR repertoire.

## Acknowledgments

We kindly acknowledge Drs. D. Colau and M. Huelsmeyer for critical discussions and support by the staff at BW6@DESY in Hamburg (Germany). We thank Karen Wagner for excellent technical support.

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