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*J Immunol* 2004; 173:6564-6573; doi: 10.4049/jimmunol.173.11.6564
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The Solvent-Inaccessible Cys\textsuperscript{67} Residue of HLA-B27 Contributes to T Cell Recognition of HLA-B27/Peptide Complexes\textsuperscript{1}

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Crystallographic studies have suggested that the cysteine at position 67 (Cys\textsuperscript{67}) in the B pocket of the MHC molecule HLA-B*2705 is of importance for peptide binding, and biophysical studies have documented altered thermodynamic stability of the molecule when Cys\textsuperscript{67} was mutated to serine (Ser\textsuperscript{67}). In this study, we used HLA-B27.Cys\textsuperscript{67} and HLA-B27.Ser\textsuperscript{67} tetramers with defined T cell epitopes to determine the contribution of this polymorphic, solvent-inaccessible MHC residue to T cell recognition. We generated these HLA-B27 tetramers using immunodominant viral peptides with high binding affinity to HLA-B27 and cartilage-derived peptides with lower affinity. We demonstrate that the yield of refolding of HLA-B27.Ser\textsuperscript{67} molecules was higher than for HLA-B27.Cys\textsuperscript{67} molecules and strongly dependent on the affinity of the peptide. T cell recognition did not differ between HLA-B27.Cys\textsuperscript{67} and HLA.B27.Ser\textsuperscript{67} tetramers for the viral peptides that were investigated. However, an aggrecan peptide-specific T cell line derived from an HLA-B27 transgenic BALB/c mouse bound significantly stronger to the HLA-B27.Cys\textsuperscript{67} tetramer than to the HLA-B27.Ser\textsuperscript{67} tetramer. Modeling studies of the molecular structure suggest the loss of a SH . . . \pi hydrogen bond with the Cys\textsuperscript{67}—Ser substitution in the HLA-B27 H chain which reduces the stability of the HLA-B27/peptide complex. These results demonstrate that a solvent-inaccessible residue in the B pocket of HLA-B27 can affect TCR binding in a peptide-dependent fashion. The Journal of Immunology, 2004, 173: 6564–6573.

\textsuperscript{1} This work was supported by the Deutsche Forschungsgemeinschaft (DFG): Ap 82/2-1 and Ap 82/2-2 (to H.A. and J.S.), Sonderforschungsbereich 421 Project C1 (to W.K.), Sonderforschungsbereich 633, Klinische Forschergruppe 104, and by the Arthritis Research Campaign U.K. (to S.Ko.) and the Medical Research Council (to W.K.), Sonderforschungsbereich 633, Klinische Forschergruppe 104, and by the Arthritis Research Campaign U.K. (to S.Ko.) and the Medical Research Council (to W.K.).

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Received for publication June 4, 2004. Accepted for publication August 20, 2004.

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\textsuperscript{3} H.A. and W.K. contributed equally to this work.

\textsuperscript{4} Abbreviations used in this paper: AS, ankylosing spondylitis; RT, room temperature; ILT, Ig-like transcript.

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a drastically altered peptide conformation in B*2705 compared with that in HLA-B*2709.

Two recent studies addressed the influence of Cys\(^{67}\) on the peptide-binding properties of HLA-B27 by performing functional analysis of the interaction between HLA-B27 and peptide. The peptide elution profile from HLA-B27 with mutant Ser\(^{67}\) was compared with nonmutant HLA-B27 molecules. These studies suggested that Cys\(^{67}\) plays a critical role in controlling the thermodynamic stability of soluble HLA-B27 molecules but that the destabilization of this molecule through the Cys\(\rightarrow\)Ser substitution is not accompanied by an alteration of the peptide-binding specificity (25). This observation differs from the results of another study showing that HLA-B27 peptide ligands failed to bind to mutant HLA-B27 molecules with serine at position 67. Instead, this molecule bound peptides not being found in nonmutated HLA-B27 molecules (30). However, the relevance of the latter seems to be more relevant because more than 1000 peptides that are naturally bound to either one or both of these molecules were analyzed compared with four peptides in the first study. It was also shown in that study that TCR binding of HLA-B27/peptide complexes was altered by using alloreactive CTLs in cytotoxicity assays suggesting that the Cys\(\rightarrow\)Ser mutation may weaken the interaction between B pocket and peptide (30). In such cellular systems it is difficult to discriminate between an effect of the Cys at position 9, and lower numbers for preferred amino acids. Peptides with scores of \(>27\) (31) and \(>1500\) (BIMAS) were regarded as high affinity ligands.

### T cell lines

Human peripheral blood cells were stimulated with 5 \(\mu\)g/ml EBV EBNA 258–266 peptide (32) in the presence of 20 \(\mu\)g/ml IL-2, 10 ng/ml IL-7, and 10 ng/ml IL-15 in T cell medium containing 10% human serum (PAA Laboratories, Cölbe, Germany), penicillin, and glutamine (Sigma-Aldrich, Taukirchen, Germany) for 2 wk. Cytokines were added at day 2 and afterward every 3–4 days.

Murine splenocytes primed in vivo with the aggrecan peptide were re-stimulated in vitro with 10 \(\mu\)g/ml of the respective peptide. The murine splenocytes were cultured at 37°C and 5% CO\(_2\) in RPMI 1640 culture medium, supplemented with 10% FCS (Invitrogen Life Technologies, Karlsruhe, Germany), 2 mM l-glutamine, 100 U/ml penicillin, and 100 \(\mu\)g/ml streptomycin sulfate and 5 \(\times\) 10\(^{-5}\) M 2-ME (Sigma-Aldrich).

### Refolding of rHLA-B27 molecules

The refolding of HLA-B27 molecules (HLA-B27 heterotrimers consisting of HLA-B27 H chain, \(\beta_2\)-microglobulin, and peptide) was performed at 4°C for 48 h in 200 ml of refolding buffer containing 100 mM Tris, pH 8.3, 400 mM l-arginine, 2 mM EDTA, and 20% glycerol. \(\beta_2\)-microglobulin and HLA-B27 H chain in an injection buffer consisting of 3 M guanidine hydrochloride (GuHCl), pH 4.2, 10 mM sodium acetate, and 10 mM EDTA and the respective peptides were added to the refolding buffer. Following refolding, proteins were concentrated in a Vivaspin concentrator (Vivascience, Hannover, Germany) against 50 mM bicine, pH 8.3. Subsequent

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### Table I. Sequence and binding scores of peptides to HLA-B2705

<table>
<thead>
<tr>
<th>Name of Peptide</th>
<th>Sequence of Peptide</th>
<th>Binding Score</th>
<th>Binding Score</th>
<th>Refolding B27.Cys(^{67}) (%)</th>
<th>Refolding B27.Ser(^{67}) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-B27/EBNA 258–266 (EBV)</td>
<td>RRYLDLIEL</td>
<td>28</td>
<td>2000</td>
<td>61.8</td>
<td>88.2</td>
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<tr>
<td>HLA-B27/aggrecan peptide(^{a})</td>
<td>SRHIAFCFR</td>
<td>20</td>
<td>1000</td>
<td>17.3</td>
<td>38.4</td>
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<tr>
<td>HLA-B27/Chlamydia peptide 138(^{b}) control peptide</td>
<td>ARKLLLIDNL</td>
<td>26</td>
<td>2000</td>
<td>67.1</td>
<td>84.2</td>
</tr>
<tr>
<td>HLA-B27/influenza NP 383–391/(^{c})</td>
<td>SRYMAIIRTR</td>
<td>26</td>
<td>1500</td>
<td>52</td>
<td>ND</td>
</tr>
<tr>
<td>HLA-B27/collagen II peptide C34(^{d})</td>
<td>DRASFIRNL</td>
<td>25</td>
<td>60</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>HLA-A2/BLF1 lytic Ag peptide 280–288 (EBV)(^f)</td>
<td>GLCTLVAML</td>
<td>29</td>
<td>6000</td>
<td>92.9</td>
<td>92.9</td>
</tr>
</tbody>
</table>

\(^a\) SYFFEITHL, University of Tübingen, Tübingen, Germany, Rammensee et al. (31).

\(^b\) HLA-peptide binding motifs (BIMAS (http://bimas.dccc.nih.gov/molbio/hla_bind/)).

\(^c\) Brooks et al. (32).

\(^d\) W. Kuon, unpublished data.

\(^e\) Kuon et al. (11).

\(^f\) Zhou et al. (31).

\(^g\) Tussey et al. (39).
concentration was performed with a Vivaspin 6 concentrator (cutoff 10,000 Dal; Vivascience) down to 1 ml. The yield of HLA-B27.Cys67 and HLA-B27.Ser67 refolding with different peptides (Table I) was analyzed by HPLC gel filtration in PBS, pH 7.4 (Superose 12, Äkta Basic System; Amersham Biosciences, Freiburg, Germany). The product peak was identified by SDS-PAGE at the elution volume of ~13.7 ml. The yield of correctly folded HLA-B27 molecules was determined by integration of the area below the elution profile using the Unicon software (version 4; Amersham Biosciences). The peak area between 7.8 and 15.9 ml was defined as 100%.

Peaks eluted after 16 ml were excluded from analyzing the percentage of refolded proteins because SDS-PAGE analysis did not detect any proteins in these fractions. The elution profile was analyzed by using Unicon Software (version 4; Amersham Biosciences). For refolding of an HLA-A2 molecule (a kind gift of Dr. K. H. Lee, Department of Hematology and Oncology, Charité Berlin, Campus Benjamin Franklin), we used an immuno-nodominant HLA-A2-restricted epitope from EBV (Table I).

**FACS analysis of CD8+ T cells with HLA-B27 tetramers**

HLA-B27 tetramers were generated as previously described (21) with some modifications. The expression vector pLMI-HLA-B27 was modified by tagging a BirA recognition site sequence as previously described (33). We used a wild-type HLA-B27 H chain with cysteine at position 67 and a mutated HLA-B27.Ser67 H chain for generating HLA-B27 molecules (21). For studying TCR binding of T cell epitopes presented by both HLA-B27 tetramers, three peptides were used: 1) EBV EBNA 258–266 (32), 2) an aggrecan-derived peptide (W. Kuo, unpublished data), and 3) a Chlamydia trachomatis-derived peptide no. 138 (11; Table I). The tetramers containing the latter peptide were used for control staining experiments. Soluble and refolded HLA-B27/peptide complexes were purified following biosynthesis by HPLC gel filtration (Äkta Basic; Amersham Biosciences) and further analyzed by gel electrophoresis (Bio-Rad, Munich, Germany). Tetramers were generated by adding PE-labeled streptavidin (Molecular Probes, Eugene, OR) at a molecular ratio of 1:4. For FACS analysis, fresh mononuclear cells from peripheral blood or splenocytes from HLA-B27 transgenic mice were incubated with a tetramer and allophycocyanin-labeled anti-human CD8 Ab or anti-murine CD8 Ab (BD Pharmingen, San Diego, CA) for 30 min at room temperature followed by washing twice with PBS/2% BSA and incubation with anti-human CD3 Ab (BD Pharmingen) for 30 min at room temperature. Cells were washed twice in PBS/2% BSA and resuspended in annexin V buffer (Molecular Probes) and 2.5 μl of annexin V (Molecular Probes) was added. CD8+ and tetramer-positive T cells were analyzed after gates were set on CD3-positive and annexin V-negative cells.

Analysis was done by using a BD Biosciences (San Jose, CA) FACScan flow cytometer with CellQuest software.

**Staining for T cell surface markers, intracellular cytokines, and analysis by flow cytometry**

T cells were stained after in vitro stimulation as described before (34). Briefly, cells from whole peripheral blood were washed with PBS/BSA, centrifuged, and stained for the CD8- and CD69-surface markers and for the intracellular cytokine IFN-γ. All stainings were performed in FACScan Permeabilizing Solution (BD Biosciences, Heidelberg, Germany). To avoid nonspecific binding of Abs to FcRs, staining was done in the presence of 1% BSA (Centeon Pharma, Berlin, Germany). The following Abs were used: anti-human CD8 PerCP (clone Leu-3A; BD Biosciences), anti-CD69 PE (Leu-23; BD Biosciences), and anti-IFN-γ coupled to Cy5 (Amersham Pharmacia Biotech, Freiburg, Germany). Positive cells were subsequently quantified by flow cytometry using a FACScanCalibur from BD Biosciences with CellQuest Software. After gating on CD8+ T cells, only cytokine-positive T cells which were also positive for the early activation surface Ag CD69 were counted.

CD8+ T cells were regarded as positive after Ag-specific staining as judged by the percentage of CD69/cytokine double-positive cells if the gated CD8+ T cells were positive without background staining (stimulation with anti-CD28 without Ag only) (34). CD69 is an early T cell activation marker and is up-regulated shortly after stimulation with specific Ags. The specificity of intracellular cytokine staining is increased by excluding cytokine+/CD69- T cells (nonspecific staining of the intracellular cytokines) from analysis.

In situ staining of Ag-specific CD8+ T cells with HLA-B27 tetramers

Sections from murine spleen were taken from HLA-B27 transgenic BALB/c mice and BALB/c mice immunized with aggrecan peptide. In situ staining was done with HLA-B27.Cys67 aggrecan peptide-, HLA-B27.Ser67 aggrecan peptide-, HLA-B27.Cys67 Chlamydia peptide no. 138-, HLA-B27.Ser67 Chlamydia peptide no. 138-, HLA-B27.Cys67 EBV peptide-, and HLA-B27.Ser67 EBV peptide tetramers. Tissue from the murine spleen was embedded in Tissue Tek and snap-frozen in liquid nitrogen. Seven-micrometer sections were prepared on a microtome at ~15°C; nonfixed sections dried for 2 min at room temperature (RT) with subsequent rehydration in PBS. Sections were further blocked for 20 min in a blocking solution containing 4% milk powder solution, anti-mouse FcγR Ab (clone 2.4G2/275; a kind gift of German Rheumatology Research Center, Berlin, Germany), and streptavidin from streptavidin/biotin blocking kit (Vector Laboratories, Burlingame, CA), followed by washing in PBS and incubation in biotin for 15 min (streptavidin/biotin blocking kit; Vector Laboratories). After washing in PBS, HLA-B27 tetramers (50 μg/ml in PBS) were incubated for 1 h at RT; tetramer-binding T cells were further labeled with a goat anti-PE (1:500 in milk powder; Biomedia, Foster City, CA) and detected by Cy3-labeled anti-goat IgG1 Ab (1:100 in PBS; Dianova, Hamburg, Germany). For CD8 staining, sections were incubated with an allophycocyanin-labeled anti-mouse CD8 Ab (1:100 in milk powder; BD Biosciences) for 1 h at RT and detected by a rabbit anti-allophycocyanin Ab (30 min, 1:500; Biozol, Munich, Germany) and a Cy2-labeled anti-rabbit IgG1 Ab (30 min, 1:300; Dianova). Cells were then dried for 15 min in 6-diamidino-2-phenylindole (DAP1) (1:300; Roche, Penzberg, Germany).

Sections were analyzed with an immune fluorescence microscope (Olympus, Hamburg, Germany). Pictures were taken with a digital camera (Olympus) and further analyzed by analysis software (Soft Imaging System, Muenster, Germany) and a Cy2-labeled anti-rabbit IgG1 Ab (30 min, 1:300; Dianova). Individual scans were analyzed using TCS-NT software and Adobe Photoshop (Adobe Systems, Mountain View, CA).

**Staining of Ig-like transcript (ILT)-2 baf cells with HLA-B27.Cys67 and Ser67 tetramers**

ILT-2 receptor binds MHC class I molecules (35) and delivers a negative signal that inhibits killing by NK cells and T cells. ILT-2 receptor transfectected and nontransfected baf cells (a kind gift from L. Lanier, University of California, San Francisco, CA) were incubated with tetramers at 37°C for 20 min in 50 μl of RPMI 1640 supplemented with 10% FCS. Cells were then washed twice on ice with FACS wash buffer (PBS, 0.5% w/v BSA, 0.02% w/v sodium azide) and fixed before FACS analysis. To determine the effect of Cy5+→Ser67 substitution on the stability of the HLA-B27 peptide complex, we incubated the HLA-B27.Ser67 aggrecan peptide and HLA-B27.Cys67 aggrecan peptide tetramers at 37°C for 1, 6, and 24 h and performed FACS analysis with ILT-2-transfected cells at a concentration of 1.5×10^6 cells/250,000 cells in 50 μl. The first staining at 0 h was performed at room temperature.

**Homology modeling of the B pocket of HLA-B27**

Based on the structure of HLA-B*2705/peptide m9 (36), the Cys67 residue was mutated manually using the program O (37). Distances and angles were determined with O as well. Fig. 6 was generated by using Molscript (Avatar Software, Stockholm, Sweden) (38).

**Results**

Refolding of HLA-B27.Cys67 and HLA-B27.Ser67 molecules with peptides of different binding affinity to HLA-B27

Tetramers of HLA-B27/peptide complexes have been made with HLA-B27 H chains in which Cys67 has been substituted with serine, in an effort to reduce aggregation based on disulfide bond formation. It has been assumed that this substitution would not affect TCR binding, given the location of this residue within the B pocket and the rather conservative nature of this substitution. Given the published studies indicating a reduced thermal stability of HLA-B27/peptide complexes with the Cys→Ser substitution, we decided to examine the feasibility of generating HLA-B27 tetramers with the native cysteine at position 67, in particular for peptides with potential relevance to the pathogenesis of AS, With
the EBV peptide, a well-described immunodominant peptide (32) with a high binding score to HLA-B2705 (score 28 (31) and score 2000 in BIMAS), the yield of refolding was efficient for both HLA-B27.Cys$^{67}$ and Ser$^{67}$ molecules: 88.2% for HLA-B27.Ser$^{67}$ and 61.8% for HLA-B27.Cys$^{67}$ (Table I, Fig. 1).

Refolding was also observed with the aggrecan peptide which has lower peptide binding scores in both computer algorithms (score 20 (31), score 1000 in BIMAS). The yield of refolded protein was $\sim$17% with the HLA-B27.Cys$^{67}$ H chain (Fig. 1) and 38.4% with the mutated HLA-B27.Ser$^{67}$ H chain (38.4%). As a control for FACS analysis and in situ tetramer staining we also generated an HLA-B27 tetramer with a C. trachomatis-derived peptide (peptide no. 138, (11)) with a binding score (score 26 in Ref. 31, score 2000 in BIMAS) intermediate between EBV peptide and aggrecan peptide. These data demonstrated that HLA-B27 H chains with the native Cys$^{67}$ could be refolded with $\beta_2$-microglobulin and peptide, even though the yield was lower than for the mutated HLA-B27 Ser$^{67}$ H chain due to a higher extent of aggregation (Fig. 1).

The reliability of the refolding assay and its correlation to peptide-binding affinity was further documented by refolding assays with an HLA-A2 molecule, which is widely used in MHC class I tetramer technology, and an immunodominant peptide from EBV (39) with high-binding affinity (29 (31) and 6000 (BIMAS)) (Table I). The yield of refolded MHC molecule was 92.9%. Another HLA-B27-restricted viral peptide with a lower binding score (influenza virus NP 383–391, binding score 26 (31), 1500 (BIMAS)) gave a lower yield of refolding (52.1%). Refolding with a peptide from collagen II, C34 (P. Atagunduz, unpublished data) revealed a low yield of refolded HLA-B27 molecules supporting the low binding score given by one computer algorithm (BIMAS).

Staining of ILT-2-transfected baf cells with HLA-B27.Cys$^{67}$ and Ser$^{67}$ tetramers

We used a biological test to address the question of whether serine for cysteine mutation changes the stability of HLA-B27 molecules and affects HLA-B27 binding to ILT-2-transfected baf cells (35). ILT-2-transfected baf cells were incubated with HLA-B27.Cys$^{67}$ and Ser$^{67}$ tetramers. Binding of both HLA-B27 tetramers refolded with EBV and aggrecan peptide was documented by FACS staining indicating that all refolded complexes are structurally intact HLA-B27/peptide molecules linked to streptavidin (tetramers) (data not shown). We next studied the stability of these complexes after different periods of time upon heating to 37°C. The staining intensity with the HLA-B27.Ser$^{67}$ aggrecan peptide tetramer was already significantly reduced after 1 h of preincubation at 37°C compared with the staining at 0 h at room temperature and further decreased depending on the preincubation time (Fig. 2). The ILT-2 staining with HLA-B27.Cys$^{67}$ aggrecan peptide tetramers also demonstrates the presence of active HLA-B27/peptide molecules because homodimeric HLA-B27 molecules would not bind to ILT-2 receptors.

FACS analysis of HLA-B27-restricted CD8$^+$ T cells with HLA-B27.Cys$^{67}$ and HLA-B27.Ser$^{67}$ tetramers loaded with aggrecan peptide

In this experiment we addressed the question of whether the substitution of serine for cysteine at position 67 in the HLA-B27 H chain influences T cell recognition. HLA-B27 transgenic mice were immunized with the aggrecan peptide and intracellular cytokine (IFN-$\gamma$) staining was performed on splenocytes after 6 h of in vitro aggrecan peptide-specific restimulation. IFN-$\gamma$-secreting CD8$^+$ T cells (0.35%) could be detected as being aggrecan peptide

* Aggregation  ○ Product peak: HLA-B27/peptide molecule

FIGURE 1. Refolding of HLA-B27.Ser$^{67}$ and HLA-B27.Cys$^{67}$ molecules. Gel filtration analysis of refolded and biotinylated HLA-B27.Cys$^{67}$ (upper three panels) and HLA-B27.Ser$^{67}$ (lower three panels) molecules loaded with aggrecan peptide (left panels), EBV EBNA 258–266 peptide (middle panels), and Chlamydia peptide no. 138 (right panels).
specific (Fig. 3, day 1, upper panels). Stimulation with anti-CD28 or control peptide (Chlamydia-derived peptide no. 138) did not induce any IFN-γ-secreting T cells.

When tetramer staining was done with the same murine splenocytes, 0.16% tetramer-binding CD8+ T cells (gates were set on CD3+ T cells) could be detected with the HLA-B27.Cys67 tetramers while 0.12% bound to HLA-B27.Ser67 tetramers with a lower shift in the histogram (Fig. 3, day 1, lower left panels). The experiment was repeated in two other equally treated mice resulting in 0.12 and 0.10% HLA-B27.Cys67 aggrecan peptide tetramer-positive CD8+ T cells and 0.10% and 0.12% HLA-B27.Ser67 aggrecan tetramer-positive CD8+ T cells, respectively. The same staining was repeated after these T cells had been stimulated in vitro with the aggrecan peptide for 7 days. By using intracellular cytokine staining, 6.53% of aggrecan-specific IFN-γ-secreting CD8+ T cells could be detected (Fig. 3, day 7, upper panels).

Anti-CD28 Abs alone or the control peptide did not induce any T cell stimulation. Comparison of Cys67 and Ser67 HLA-B27 tetramers with this aggrecan peptide-specific T cell line revealed an important result (Fig. 3, day 7, lower right panel): while HLA-B27.Cys67 tetramers detected only a minority of aggrecan peptide-positive CD8+ T cells (0.86%) with a low shift in the histogram, HLA-B27.Cys67 tetramers detected 4.13% aggrecan tetramer-positive CD8+ T cells, respectively. The same staining was repeated after these T cells had been stimulated in vitro with the aggrecan peptide for 7 days. By using intracellular cytokine staining, 6.53% of aggrecan-specific IFN-γ-secreting CD8+ T cells could be detected (Fig. 3, day 7, upper panels). The specificity of tetramer staining was confirmed by intracellular cytokine staining of IFN-γ-secreting and CD69+ T cells (41.14%) after peptide-specific T cell stimulation (Fig. 4, day 14, upper panels).

FACS analysis of HLA-B27-restricted CD8+ T cells with HLA-B27.Cys67 and HLA-B27.Ser67 tetramers loaded with a high affinity immunodominant EBV peptide

We repeated the above-mentioned experiments with EBV EBNA 258–266-specific CD8+ T cells to determine whether T cell recognition of the MHC/peptide complex is also altered for peptides with a higher affinity. Peripheral blood was taken from an HLA-B27+ blood donor with previous EBV infection. Intracellular cytokine staining revealed 0.84% IFN-γ-secreting CD8+ T cells after 6 h of EBV EBNA 258–266 peptide-specific in vitro stimulation (Fig. 4, day 1, upper panels). FACS staining with the HLA-B27.Cys67 and HLA-B27.Ser67 tetramers showed a comparable result of Ag-specific T cell detection (Fig. 4, day 1, lower panels). The same experiment was repeated with an EBV peptide-specific T cell line (Fig. 4, day 14, lower panels). HLA-B27.Cys67 and HLA-B27.Ser67 tetramers again detected similar numbers of EBV-specific CD8+ T cells, 76.69% tetramer binding CD8+ T cells with the HLA-B27.Cys67 tetramer, and 79.89% with the HLA-B27.Ser67 tetramer. The finding that similar populations of CD8+ T cells were labeled with both tetramers was reproducible in multiple experiments with the shown T cell line, and also with other T cell lines of the same specificity (data not shown). The specificity of tetramer staining was confirmed by intracellular cytokine staining of IFN-γ-secreting and CD69+ T cells (41.14%) after peptide-specific T cell stimulation (Fig. 4, day 14, upper panels).
In situ tetramer staining of HLA-B27-restricted CD8\(^+\) T cells in the spleen of HLA-B27 transgenic BALB/c mice

To compare T cell recognition of both HLA-B27 tetramers loaded with the aggrecan peptide in situ, we examined snap-frozen spleens (40) from aggrecan-peptide immunized HLA-B27 transgenic BALB/c mice, nonimmunized HLA-B27 transgenic BALB/c mice and aggrecan peptide-immunized and nonimmunized BALB/c mice. From FACS analysis of tetramer staining of murine splenocytes a frequency of up to 0.3% tetramer-positive T cells among CD8\(^+\) T cells in the spleen was expected.

In aggrecan peptide-immunized HLA-B27 transgenic BALB/c mice, we identified aggrecan peptide-specific CD8\(^+\) T cells in these in situ staining experiments. As shown in Fig. 5 tetramer-binding cells could be detected when HLA-B27.Cys\(^{67}\) tetramers with the aggrecan peptide were used (Fig. 5B), but not when HLA-B27.Ser\(^{67}\) tetramers with the aggrecan peptide were used (not shown). When double staining with anti-CD8 Ab (green) and HLA-B27.Cys\(^ {67}\) tetramer (red) was performed, double-positive cells could be detected (yellow) (Fig. 5C). In this experiment, all tetramer-positive T cells were also CD8\(^+\) T cells. Using the HLA-B27.Ser\(^{67}\) tetramer with the aggrecan peptide did not provide any in situ detection of aggrecan peptide-specific CD8\(^+\) T cells (not shown). HLA-B27.Cys\(^{67}\) tetramers loaded with the aggrecan peptide bind to splenocytes (data not shown). These experiments demonstrate that in the case of in situ detection, T cell recognition of Ag-specific CD8\(^+\) cells with HLA-B27.Ser\(^{67}\) tetramers is completely abolished.

Impact of Cys\(^{67}\) for the coordination of pR2 in the B pocket

Various crystal structures of HLA-B*2705 demonstrate that the B pocket is sterically and electrostatically ideally suited to accommodate an arginine side chain (Fig. 6). Its hydrophobic part forms van der Waals contacts to Tyr\(^7\) and Ile\(^{66}\), while the charged guanidyl moiety is stabilized by a bifurcated salt bridge to Glu\(^{45}\) and a hydrogen bond to Thr\(^{24}\). The side chain of Cys\(^{67}\) (Fig. 6B) points directly toward the pR2 guanidyl group and fulfills the geometric requirements to qualify as an SH ... H9266 hydrogen bond (\(\omega = \angle \text{SH ... H9266} < 25^\circ\) [20\(^\circ\)] and distance S ... H9266 < 4.0 Å [3.5 Å]) (41). This type of hydrogen bond is rarely found and is suggested to be weaker than classical hydrogen bonds (41). Mutation of Cys\(^{67}\) to Ser\(^{67}\) abrogates this interaction (Fig. 6B) as an intact OH ... H9266 hydrogen bond should not be longer than 3.8 Å (41), but is actually found to be 4.1 Å due to the shorter C-O bond distance in the serine side chain. Instead, only a small reorientation of roughly 25\(^\circ\), the Ser\(^ {67}\) hydroxyl group can engage in a standard hydrogen bond (2.8 Å) to the backbone oxygen of Glu\(^{63}\). Based on the hard soft acid base concept, this interaction is clearly favored over a weak OH ... \(\pi\) hydrogen bond.
Discussion

By using HLA-B27 tetramer technology we demonstrate that a substitution of residue Cys67 to Ser67 in the B pocket of the HLA-B27 H chain affects T cell recognition in a peptide-dependent fashion.

The alteration of peptide presentation and reduction of T cell recognition as a consequence of Ser67 substitution is currently a matter of discussion (25, 30). Our results indicate that an alteration of T cell recognition by HLA-B27.Ser67/peptide complexes is present which is strongly dependent on the peptide bound to the HLA-B27 molecule. This conclusion is based on refolding of HLA-B27 molecules with Cys67 and Ser67 H chains with different peptides and the use of such molecules for tetramer staining of peptide-specific CD8+ T cells by in situ and FACS analysis.

In our studies, we used a human aggrecan-derived peptide (W. Kuon, unpublished data) to immunize HLA-B27 transgenic BALB/c mice to obtain CD8+ T cells with the respective specificity. FACS staining with HLA-B27.Ser67 and HLA-B27.Cys67 tetramers of murine CD8+ T cells, which were generated in vitro from splenocytes of these mice, revealed significantly reduced numbers of tetramer-positive CD8+ T cells by in situ and FACS analysis.

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These findings raise the question of whether the differences in T cell recognition can be correlated to different binding modes of pR2 as a consequence of the mutated residue 67 in the B pocket. In fact, a drastic alteration in the peptide presentation mode caused by a single buried and polymorphic residue was recently described (10). However, the situation is different in the situation discussed here. The Cys67→Ser substitution probably leads to the loss of a peculiar SH...hydrogen bond and the formation of a new intrahelical interaction (Fig. 6). Even though this alteration leads to destabilization of the HLA-B27/peptide complex as shown by reduced melting temperatures for the HLA-B27.Ser67 variant (25), we do not think that a grossly different peptide conformation is present. The remaining interactions are unchanged, and the lost SH...π bond is comparably weak, suggesting that the pR2 anchor is still securely bound in the B pocket.

These results indicate that presentation of the aggrecan peptide to CD8+ T cells is fundamentally altered when cysteine is substituted by serine at residue 67. We could not perform the same experiment with HLA-B27.Ser67 transgenic mice to see whether this difference would be the same. However, we would have expected the same result. In contrast, by using a well-described immunodominant EBV-derived peptide, Ag presentation through HLA-B27.Ser67 was not significantly different from HLA-B27.Cys67 because HLA-B27 tetramer staining with both molecules revealed almost identical numbers of Ag-specific CD8+ T cells.

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The immunodominant EBNA peptide is present. In this case the pR1 residue stabilizes the B27.Ser67 molecule and compensates for the flexibility resulting from the B pocket mutation. The structural explanation of the thermodynamic stabilization effect is a stacking interaction of pR1 with W167 and R62 (42).

In line with this finding is the observation of the peptide repertoire eluted from HLA-B*2705 which shows a preference for arginine (R) at peptide residue P1 (43). Only in those cases, where p1 is not an arginine, the destabilized B pocket becomes “visible” at the protein surface in terms of differences in the T cell response as seen in our study for those complexes loaded with the aggrecan peptide.

Indeed, it has been proposed that the stability of the MHC-peptide complex is more important for T cell binding and T cell stimulation than peptide-binding affinity (44). We next used a biological test to address the question of whether a loss of stability of the HLA-B27/peptide complex could explain our results. HLA-B27 tetramers refolded with Ser67 and Cys67 H chains bound to baf cells transfected with an ILT-2 receptor, which binds to MHC class I molecules (35). After preincubation at 37°C the binding of the HLA-B27.Ser67 aggrecan peptide tetramer was significantly reduced after 1 h suggesting that the staining of ILT-2-transfected cells strongly depends on the stability of the HLA-B27 complex. These findings are in line with the hypothesis that the stability of the molecule might have been altered by the mutation at position 67 of the HLA-B27 H chain (25), which might also be the reason for an abrogation of mAb recognition of HLA-B27 molecules after site-directed mutagenesis of residue 67 Cys to tyrosine (45, 46).

Further support is given by recent thermodynamic studies demonstrating for HLA-B*2705 and HLA-B*2709 that a single amino acid exchange at residue 116 can have large effects on the stability of these two HLA-B27 molecules (42).

The critical mechanisms for T cell recognition of MHC/peptide complexes are currently discussed controversially and there is some evidence that structural rearrangements or flexibility during the binding of MHC/peptide complexes to the TCR contribute to T cell activation (47). Our studies also indicate that in addition to structural properties, biophysical properties like thermodynamic stability may also influence T cell recognition of the MHC/peptide complex. More experiments are necessary to elucidate the precise molecular mechanisms by which the Ser67 mutation affects stability and peptide presentation by HLA-B27.

It has been speculated that the HLA-B27 association to AS is not related to Ag presentation but rather to a misfolding of HLA-B27 H chains or triggering of stress responses from the endoplasmatic reticulum (23, 48). In vitro experiments with HLA-B27 H chains revealed the presence of disulfide-linked HLA-B27 homodimers and stimulation of T cell responses by these homodimer molecules (22, 49, 50). We can rule out that tetramer staining in our experiments was caused by HLA-B27 homodimers because we did not detect any CD8" T cells when HLA-B27.Cys67 tetramers were used with a control peptide. In addition, the presence of peptide-specific CD8" T cells was confirmed by intracellular cytokine staining for IFN-γ-producing cells after peptide stimulation. Moreover, we confirmed the presence of the peptide in the HLA-B27-binding groove by HPLC excluding that the absence of tetramer
FIGURE 6. Impact of residue 67 on the coordination of pR2 in the B pocket of HLA-B27. A. Overall topological view into the peptide-binding groove based on HLA-B*2705:m10 (36). Secondary structure is depicted in gray, the peptide is shown as a stick representation. Close-up views into the B pockets of B*2705.Cys67 (B) and B*2705.Ser67 (C). Oxygen atoms are shown in red, nitrogen in blue, sulfur in yellow, carbon atoms from the H chain are depicted in gray, from the peptide in green. The pR2 side chain is stabilized by several polar interactions (black dotted lines). A rare SH . . . π bond (orange dotted line) between the cystine and the Arg guanidyl head group contributes to the hydrogen bonding network. Mutation of Cys67 to Ser is proposed to establish an altered network with Ser67ORH contacting E63O3 (blue dotted line).

staining was caused by the fact that the aggrecan peptide did not bind to the HLA-B27-binding groove (data not shown).

What are the consequences for generating HLA-B27 tetramers based on these observations? The use of HLA-B27 tetramers was first described by studying critical binding sites of HLA-B27-restriction T cell receptors (21). For these experiments, mutant HLA-B27.Ser67 molecules loaded with an immunodominant peptide from influenza virus (51) were successfully used. We have also used HLA-B27.Ser67 tetramers effectively to study HLA-B27-restricted T cell epitopes in C. trachomatis-triggered reactive arthritids (52). Although the yield of refolded HLA-B27 molecules is higher with Ser67 H chain and T cell staining with HLA-B27.Ser67 tetramers is not abrogated for certain peptides, our data indicate that HLA-B27.Cys67 tetramers may permit identification of T cell populations that could be missed when HLA-B27.Ser67 tetramers are used.

Acknowledgments
We thank Sabine Seibert and Ulrike Erben for performing the analysis of tissue sections by confocal microscopy.

References


