**T Cell Receptor Selection by and Recognition of Two Class I Major Histocompatibility Complex-restricted Antigenic Peptides That Differ at a Single Position**

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**Summary**

Peptides derived from HLA-Cw3 and HLA-A24 within region 170-179 differ by a single substitution, at position 173, and are both presented by the class I major histocompatibility complex molecule H-2Kd for recognition by murine cytolytic T lymphocytes (CTLs). As a first approach to understand the way T cell receptors (TCRs) interact with the HLA peptides, we have analyzed the TCR selection by, and recognition of, the two HLA antigenic sites. First, we have compared the TCR repertoires selected by HLA-Cw3 and HLA-A24, not only by sequencing the TCRs carried by CTL clones isolated and grown in vitro, but also by analyzing the TCRs expressed in vivo by peritoneal exudate lymphocytes from immune animals. Second, we have compared the TCR crossrecognition of HLA-A24 by CTLs selected by HLA-Cw3 with that of HLA-Cw3 by CTLs selected by HLA-A24. The combined analysis of TCR selection by and recognition of these two related HLA antigenic sites provides evidence that the TCR β junctional regions interact with the amino-terminal part of the HLA peptides.

**CTLs recognize antigenic peptides presented by class I MHC molecules** (1, 2). The specificity of this recognition is conferred by the TCR α/β (3, 4). Whereas the structure of several class I MHC molecules, and that of a class I MHC-peptide complex, were determined by crystallographic studies (5), such information is not available yet for the TCR. However, a model of the TCR α/β tertiary structure was proposed, based on its homology with Igs (6). In any case, experimental evidence is lacking so far to support a topology of class I MHC-peptide recognition by TCRs.

We previously reported that DBA/2 mice could mount a CTL response towards two related antigens, HLA-Cw3 and HLA-A24, in the context of the same murine class I MHC molecule, H-2Kd, and that a fraction of the CTLs raised in response to either HLA antigen was not reactive to the second HLA antigen (7). The optimal synthetic peptides recognized by these specific CTLs, corresponding to the region 170-179, differ by a single nonconservative substitution at position 173 and bind their common restriction element H-2Kd with a similar affinity (Table 1) (8–12). Regions 170-179 of the related HLA molecules A2 and A3 are identical to HLA-A24 and can also be recognized by H-2Kd-restricted CTLs (13).

We recently showed that the TCRs carried by H-2Kd-restricted CTLs specific for the Cw3 170-179 peptide were very limited in primary structure (14). They were encoded by few germline gene segments: a single VB segment (ORB10), a single Jo2 segment (JcαpHDS58), few Va2 segments (mainly Va3, 4, 8), and few Jα2 segments (mainly Jα1.2, 1.4, 2.3). Their junctional CDR3 α and β loops also displayed limited diversity: a single length of nine and six amino acids, respectively, a conserved non-V-, non-J-encoded glycine amino acid at position 97 in the CDR3 β, and a high occurrence of non-J-encoded glycine or charged amino acids at positions 94 and 95 in the CDR3 α.

As a first approach to understand the topology of interaction of TCRs with their class I MHC-peptide ligand, we have now taken advantage both of the single amino acid difference between the Cw3 and A24 antigenic peptides and of the existence within each of the two CTL responses to HLA antigens of a noncrossreactive CTL population.

We first tested a large series of Cw3-selected CTL clones bearing TCRs of known primary structure for crossrecognition of A24. We then determined the TCR repertoire selected by the A24 peptide, not only by sequencing the TCRs carried by CTL clones isolated and grown in vitro, but also by analyzing the TCR repertoire used in vivo by peritoneal ex-
Table 1. HLA-Cw3 and HLA-A24 170–179 Antigenic Peptides

<table>
<thead>
<tr>
<th>Cw3</th>
<th>R</th>
<th>Y</th>
<th>L</th>
<th>K</th>
<th>N</th>
<th>G</th>
<th>K</th>
<th>E</th>
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<tr>
<td>A24</td>
<td></td>
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<td>-</td>
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<td>E</td>
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</table>

The HLA-Cw3 and A24 molecules differ at a single position (173) within region 170–179 (8). The HLA-A2 and -A3 molecules, as well as the recombinant molecules 2.2/3.3 and 3.3/2.3, display the same amino acid sequence as HLA-A24 (13). Both peptides harbor the Kδ binding motif (underlined) (2, 11, 12) and bind equally well to Kδ (not shown).

Materials and Methods

**CTL Clones and PELs.** The CTL clones used in this study are listed in Table 2. They were maintained in culture as described in the references. PELs were isolated and analyzed as previously described (14).

**Cell Staining and Cytolytic Assays.** The cell staining was performed as previously described, as were the cytolytic assays (14).

**Direct Sequencing of PCR Products.** The cDNA PCR and direct sequencing were performed as previously described (14-17).

**Oligonucleotides.** The JB1.2 and JB1.4 primers will be reported elsewhere (Parmentier et al., manuscript in preparation). Sequences of the newly designed primers specific for the VαT2.5–5 (18) and VαB6.2.16 subfamilies (19) are CAGAAAACAGAGCCAAAGAC and GAGACACCGTTGTTAAAGGC, respectively. The other primers were previously described (15).

Results

**Crossrecognition of A24 by CTL Clones Selected by Cw3.** We previously reported the TCR sequences of 37 independent H-2Kδ-restricted CTL clones specific for the Cw3 170–179 antigenic site (14; Casanova et al., manuscript in preparation). We have now tested 30 of them for crossrecognition of the same antigenic site in A24.

As summarized in Fig. 1, where the TCR primary structures are shown along with the peptide fine specificity, most anti-Cw3 CTL clones are affected by the substitution at position 173. 11 clones do not recognize A24 at all, and 16 clones display an "intermediate" recognition profile of A24. Among the latter category, the patterns were in fact heterogeneous, as illustrated in Fig. 2. Only three clones appear to recognize A24 as well as Cw3.

The usage of the only two variable segments of the TCR repertoire selected by Cw3, namely Jβ and Vα, was not evenly distributed between crossreactive and noncrossreactive clones.

The Jβ1.4 and Jβ2.3 segments were more frequent among noncrossreactive (6/11) than crossreactive (3/19) clones, whereas the reverse trend was found for Jβ1.2. The Vα3 subfamily was more frequent among noncrossreactive (5/11) than crossreactive (1/19) clones, whereas the reverse trend was found for Vα4 and Vα8.

The amino acids occurring at two positions of the CDR3 α also showed some bias with respect to crossreactivity pattern. At position 95, many (11/19) crossreactive clones expressed a non-Vγ, non-J-encoded positively charged residue, whereas none of the 11 noncrossreactive ones did. The frequency of negatively charged residues at positions 94 in the CDR3 α, mostly Vα4 and Vα8 encoded, was higher among crossreactive (16/19) than noncrossreactive (6/11) CTL clones.

An examination of the reactivity patterns of individual clones that share identical α or β chains further illustrates this uneven distribution of TCR structural features, and demonstrates that the pattern of A24 crossreaction cannot be attributed solely to either chain. Several sets of CTL clones displaying identical TCR β chains, but different reactivities, were found. Clones Cw3/4A3, HLA1G6, PEA1, and PEA34 crossreact with A24, unlike PEA13, although they share the same TCR β sequence. Notably, the former clones are all Vα8 and their CDR3 α contain a pair of complementary charges, while the latter clone is Vα3 and its CDR α contains only an acid charge. Similarly, clones Cw3/PEA30 and HLA1C8 share the same TCR β sequence and are both Vα4, but the former does crossreact and its CDR3 α contains a pair of opposite charges, whereas the latter does not crossreact and its CDR3 α contains only an acid residue. Finally, whereas the clone Cw3/C37 is Vα8, with a CDR3 α containing two complementary charges, the clone HLA2D3 is Vα4, its CDR3 α contains no charged residue, and only the former clone crossreacts although they both display the same TCR β sequence.

Conversely, CTL clones sharing an identical TCR α chain (although, rigorously, divergence in the Vα segment upstream of the region sequenced may exist), but differing in fine specificity, were also found. Clones Cw3/1.1, HLA2A3, and PEA21 express the same TCR α chain, but only the latter does not crossreact with A24. Its TCR β differs from that of the two other clones in Jβ segment usage (Jβ2.3 instead of Jβ1.2 or Jβ2.7).

Thus, in the absence of any strict structure-function segregation, it is impossible to conclude from these data that any of these TCR regions confer or block crossrecognition of A24. Rather, it appears that crossrecognition of A24 does not depend on a single region of the TCR, since several regions of the α/β heterodimer correlate with the crossreactivity patterns. Moreover, the possibility remains that any of these TCR regions may contribute to the crossreaction pattern by modulating the overall affinity of the complex, even by interacting with a region distinct from residue 173. Altogether, a firm conclusion in terms of topology of the ternary complex cannot be drawn from these first results alone. To investigate further the contribution of each of these TCR elements, we next assessed the effect of the substitution at position 173 found in A24 on the direct selection of a TCR repertoire.
Table 2. Origin of H-2K^d-restricted CTL Clones Selected by the HLA-A24 170-179 Antigenic Site Used in This Study

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Strain</th>
<th>Immunogen *</th>
<th>In vitro stimulation</th>
<th>CTL clones</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DBA/2</td>
<td>P815-A24</td>
<td>P815-A24</td>
<td>A24/10.1, 12.2</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>DBA/2</td>
<td>P815-A3</td>
<td>P815-A3</td>
<td>A3/74.1, 72.2</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>DBA/2</td>
<td>P815-A3/hβ2m</td>
<td>P815-A3/hβ2m</td>
<td>A3/1IC1</td>
<td>This report</td>
</tr>
<tr>
<td>4</td>
<td>DBA/2</td>
<td>P815-A3/hβ2m</td>
<td>P815-A3/hβ2m</td>
<td>A3/1IC7</td>
<td>This report</td>
</tr>
<tr>
<td>5</td>
<td>DBA/2</td>
<td>P815-A3/hβ2m</td>
<td>P815-A3/hβ2m</td>
<td>A3/IIIC5</td>
<td>This report</td>
</tr>
<tr>
<td>8</td>
<td>DBA/2</td>
<td>P815-A2</td>
<td>P815-A2</td>
<td>A2/25</td>
<td>13</td>
</tr>
<tr>
<td>10</td>
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<td>P815-2.2/3.2</td>
<td>P815-2.2/3.2</td>
<td>223/5, 14, 27</td>
<td>13</td>
</tr>
<tr>
<td>11</td>
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<td>P815-2.3/3.3</td>
<td>P815-2.3/3.3</td>
<td>332/1K, 2A, 2G</td>
<td>13</td>
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<tr>
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<td>P815-A24</td>
<td>A24/PEF1, 2, 4, 5, 8</td>
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<tr>
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<td>P815-A24</td>
<td>P815-A24</td>
<td>A24/PEG2</td>
<td>This report</td>
</tr>
</tbody>
</table>

The specificity of the CTL clones from mice 1, 2, and 8-13 was established by recognition of the transfectant cell line P815-A24 (6) and was further documented with P815 cells pulsed with synthetic peptides corresponding to the region 170-182 (for clones A24/12.2 and A3/74.1) or 170-179 (all other clones) of the HLA-A24 molecule. The specificity of CTL clones from mice 3-7 was established by recognition of P815 cells transfected with HLA-A3, whose sequence in the 170-179 region is identical to that of HLA-A24. The H-2K^d restriction of the CTL clones mice 3-7 is based on antibody blocking experiments, and that of clones from mice 1, 2, and 8-13 is presumed from recognition of A24 peptides, which are known to bind to H-2K^d. * P815-A24 indicates a P815 mastocytoma cell line transfected with the HLA-A24 gene (6), and P815-A2, A3, 2.2/3.2, and 3.3/2.3 indicate P815 cell lines transfected with natural or recombinant genes encoding proteins that share A24 sequence within region 170-179 (13). P185-A3/hβ2m indicates a P185 cell line transfected with the HLA-A3 gene and the human β2m gene (Barra et al., unpublished results). * These clones were isolated as described in references 6 and 10. $ These clones were derived by limiting dilution of CD8^+ PELs, from animals immunized intraperitoneally with P815-A24 transfectant cells.

TCRs Carried by CTL Clones Selected by A24. We analyzed a collection of 26 H-2K^d-restricted CTL clones specific for the site 170-179 of HLA-A24 that were judged to be independent based on differences either in TCR nucleotide sequence or in the animal of origin (Table 2).

The TCR repertoire selected by A24 appears to be in many respects similar to the TCR repertoire selected by Cw3 (Figs. 3 and 4). In particular, most CTL clones express TCRs bearing the Vβ10 and JαpHDS58 segments. However, the A24-selected TCR repertoire also seems to be broader, as illustrated by the presence of additional structures in most regions of the α/β heterodimers.

When precisely compared with the TCRs from the Cw3-selected TCR repertoire, the TCRs selected by A24 clearly fall in three categories. The first group (I) includes 11 TCRs that are indistinguishable from those found in the Cw3-selected TCR repertoire. They express the Vβ10 segment, a Jβ1.2, 2.3, 1.1, or 2.7 segment, a Vα8, 4, 3, or 5 segment, and the JαpHDS58 segment. They display a CDR3 α length of nine amino acids, a CDR3 β length of six amino acids, a glycine or a charged residue at positions 94 and 95 in the CDR3 α, and a glycine at position 97 in the CDR3 β. The resemblance with the HLA-Cw3 TCR repertoire is such that six and four of these A24-selected CTL clones express TCR α and β chains, respectively, identical to some of those found among Cw3-selected CTL clones. The other TCRs differ from the Cw3-selected TCRs either by few conservative substitutions in either of the CDR3 or by the usage of a distinct member of the same Vα subfamily.

The second group (II) includes six TCRs, which differ from the Cw3-reactive TCRs by only a single criterion. Five clones express a CDR3 β length of 10 amino acids, all of which are Vβ10-Jβ1.4. In contrast, all Vβ10-Jβ1.4 CDR3 loops in the anti-Cw3 TCR repertoire were of six amino acids. In addition, one clone uses the Jβ2.4 segment, which was not found in the Cw3-reactive TCR repertoire. Among the TCRs of group II, one TCR α chain is identical to one found in the Cw3 repertoire, and the others differ by only a few conservative substitutions in the CDR3 or by the usage of a distinct member of the same Vα subfamily from the anti-Cw3 TCR repertoire.

The third group (III) includes nine clones, in which at least two changes were observed when compared with the TCR features characteristic of the response to Cw3. Remarkably, in all cases at least one of these changes concerns the TCR β junctional region, again either in the usage of different Jβ segments (Jβ2.4, 1.3, 2.5, 2.1), or of different CDR3 lengths (Vβ10-Jβ1.4 loops of 10 amino acids). Additional changes can affect every part of the heterodimers: Vβ segment usage, CDR3 β length, lack of glycine at position 97 in the CDR3 β, Vα subfamilies usage, CDR3 α length, and Jα segment usage.
When compared with the Cw3-selected TCRs that did not crossrecognize A24, it appears that A24-selected TCRs display paradoxically a similar distribution in terms of Vα3 subfamily usage and charged amino acid composition at positions 94 and 95 of the CDR3 α.

The frequency of Vα3 among A24-selected clones (6/26) is similar to that of Cw3-selected clones (6/30), although of the latter Vα3 clones, most did not crossrecognize A24. The frequency of negatively charged residues in the CDR3 α at position 94 is also lower (8/26 vs. 22/30), and even lower is the frequency of positively charged residues at position 95 (5/26 vs. 12/30), although again the negatively charged residue at position 94, and even more, the positively charged residue at position 95, were found to be less frequent among the anti-Cw3 clones that did not crossrecognize A24.

These results suggest that the Vα usage and the CDR3 α composition in charged residues would not be critical for recognition of position 173 of the HLA peptides, but rather would contact another region, possibly contributing to the crossreaction patterns observed among Cw3-selected clones for recognition of the P815-Cw3 and A24 transfectant cells. Clone Cw3/56.1 was tested for recognition of P815-Cw3 and A24 transfectant cells. Clones Cw3/PEA1, 9, 13, 14, 21, 23, 30, and 34 were tested for recognition of the HLA-Cw3 and A24 170-179 peptides, and for recognition of the P815-Cw3 and A24 transfectant cells.

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cross-recognition of A24 by a set of Cwb-selected CTL clones. For recognition either of P815 cells transfected with the HLA-Cw3 (O) or A24 (O) genes (left), or of P815 (H-2d) target cells pulsed with HLA-Cw3 (O) or A24 (O) 170–179 peptides (right). Lysis of control P815 cells without peptide is also shown (dashed lines).

The proportion of Vβ10 among CD8 cells was significantly increased, indicating that the Vβ10 dominance observed on CTL clones also occurred in vivo (not shown). However, among A24-selected PELs, the higher variation of the Vβ10 percentage among individuals, as well as the somewhat lower percentage on average than that found among Cwb-selected CTL clones analyzed, we then compared the TCR repertoire used in vivo in response to Cw3 and A24.

PELs were harvested after intraperitoneal immunization with P815-Cw3 and P815-A24 transfectant cells, and analyzed as previously described (14). They displayed specific cytolytic activity without any stimulation in vitro (not shown). To rule out any bias in vitro during the isolation of this set of CTL clones, or any bias due to an insufficient number of CTL clones analyzed, we then compared the TCR repertoire used in vivo in response to Cw3 and A24.

To analyze the TCR response in more detail, a first cDNA PCR with a Vβ10 and a Jβ1.2 pair of primers was performed on the Cw3- and A24-specific PELs, and the product was directly sequenced with the Jβ1.1 primer. As shown in Fig. 6, the product was not only clearly readable, indicating a homogeneity of the TCRs bearing these two segments, but also encoded a CDR3 of the same length as that of Cwb- or A24-reactive CTL clones known to express Vβ10-Jβ1.2.
TCRs. As a control, the fragments amplified from LNs of nonimmunized mice were not readable at all, reflecting the extensive diversity of Vβ10-Jβ1.2 junctions among unselected lymphocyte populations.

Moreover, the apparent distinction between the TCR repertoires from CTL clones selected by HLA-Cw3 and HLA-A24, in terms of length of the Vβ10-Jβ1.4 CDR3, was also evident in vivo, since PELs from Cw3- or A24-immunized mice displayed Vβ10-Jβ1.4 loops of mutually exclusive lengths of 6 and 10 amino acids, respectively (Fig. 6). Thus, a distinctive feature of the TCR β junctional region repertoire, obtained from the comparison of a series of Cw3- and A24-selected CTL clones isolated and grown in vitro, was found to be relevant in vivo.

These findings support the conclusions drawn from the analysis of TCR clones isolated and grown in vitro, and indicate that the TCR β junctional region may be critical in recognition of position 173 of the HLA peptides in vivo.

Discussion

For the CTL clones selected either by Cw3 or A24 that do crossrecognize the other HLA allele, interaction with position 173 does not appear to be critical to the overall reog-
Figure 4. TCR α and β chains of A24-selected CTL clones and their crossrecognition of Cw3. The 26 CTL clones are listed on the vertical axis. For each clone, the in-frame TCR β transcript encoding the key residues at the VDJ junction (6) was considered to encode the functional TCR β chain. For CTL clones A24/PEF1, 2, 4, 5, and 8, a FACS staining with the anti-Vβ10 mAb B21.5 (52) was performed to confirm the β transcript assignment. The deduced amino acid sequences of the junctional and hypervariable regions, putatively CDR3-like, are reported (in single-letter amino acid code) according to reference 6. The presumed Ig-like loops, designated CDR3 for convenience, are putatively CDR3-like, are reported (in single-letter amino acid code) according to reference 6. The presumed Ig-like loops, designated CDR3 for convenience, are putatively supported by two framework branches (FW), which are also reported here. The key Cys residue is at positions 90 and 92 in the α and β chains, respectively. The Vα, Vβ, Jo, and Jβ segments are also reported (see Fig. 3 for references). Regions of the TCR α or β chains expressed by the A24-selected CTL clones that differ from those expressed by Cw3-selected CTL clones were boxed. The α transcript can be unambiguously assessed to encode the functional α chain, i.e., paired with the β chain to form a heterodimer specific for the HLA-A24 peptide–H-2Kd complex, only when a second, out-of-frame transcript was also detected (15, 53). An out-of-frame α transcript was found in CTL clones A24/12.2, PEF4, A3/H2R2, H2R5, 72.2, and 332/2A, and a second α transcript, in-frame at the Vβ junction, was found in CTL clones A24/10.1 and 322/1K. However, for the latter two and the remaining CTL clones, the α transcripts reported here are likely to encode the functional α chains because of their structural homology with the unambiguously assigned ones. All CTL clones recognize the HLA-A24 170–179 antigenic site ( ). Level of crossrecognition of the Cw3 170–179 antigenic site is indicated: ( ) recognition of Cw3 as well as that of A24; ( ) intermediate recognition; or ( ) no recognition. Clones that were not tested for recognition of Cw3 are indicated (NT). Clones A3/74.1 and 72.2 were further tested for recognition of P815-Cw3 and A3 transfectant cells and for recognition of Cw3 and A24 170–182 peptides, and clone A3/72.2 was further tested for recognition of Cw3 and A24 170–179 peptides. Clones A24/10.1, 72.2, and 332/2A, 332/2G, A2/25, and A3/63 were tested for recognition of P815-Cw3 and A24 transfectant cells and for recognition of Cw3 and A24 170–179 peptides. E/T ratios or peptide concentrations were titrated to evaluate the relative recognition of transfectants or peptides, respectively.
These models were essentially based on the much higher variability of the CDR3 loops of the TCR would primarily interact with the antigenic peptide, whereas the CDR1 and 2 would contact the MHC restriction element. (6, 20, 21). These models were essentially based on the much higher variability of the CDR3 and antigenic peptides, when compared with the CDR1, 2, and MHC molecules, respectively. 

Experimental evidence supporting this model, and in particular that TCR CDR3 loops were critical for recognition of the antigenic peptide, has been provided in various class II MHC-restricted systems. Conclusions were mainly based on the differences of peptide fine specificities displayed by T cells bearing TCRs, either natural variants (22-25) or genetically engineered (26, 27), that differed at a single position, has been reported recently in the class II MHC-restricted response to cytochrome c (28). The results of analyzing the TCR repertoire in single chain TCR transfectant systems. The orientations may indeed differ from case to case. Additionally, elements other than the TCR sequences, which vary from cell to cell, may also affect recognition patterns, as seems to be the case in the present study among Cw3-selected CTL clones tested for crossrecognition of A24. These include level of TCR, level of adhesion molecules, sensitivity of activation pathways, etc. In addition, correlations between TCR primary structure and fine specificity may not necessarily reflect only the direct interactions of the structures involved, but also differences in affinity provided by interactions at other places. 

To settle this issue, an elegant approach, which consists of analyzing the TCR repertoire in single chain TCR transgenic mice immunized with variant peptides substituted at a single position, has been reported recently in the class II MHC-restricted response to cytochrome c (28). The results indicate that both CDR3 α and β are critical for peptide recognition.
recognition, and that the CDR3 $\beta$ would interact with the carboxy-terminal part of the peptide, whereas the CDR3 $\alpha$ would interact with the amino-terminal part of the peptide.

We have adopted a similar approach to the class I MHC-restricted response to HLA-Cw3 with two modifications. First, we have immunized normal mice, which were free to adapt either of the TCR chains in response to the single amino acid substitution. Second, we have analyzed the TCR repertoire used in vivo by harvesting PELs obtained from immune animals in order to confirm the findings obtained on T cell clones isolated and grown in vitro.

Altogether, our findings provide evidence that the TCR $\beta$ junctional regions interact with the amino-terminal part of the HLA peptides presented by a class I MHC molecule. Notably, this orientation differs from that found in the class II MHC-restricted response to cytochrome c (28). Further experiments are required to determine whether the orientation of the TCRs on the surface of MHC/peptide complexes varies according to the class of the MHC or to the nature of the peptide.

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