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# Peptide Contributes to the Specificity of Positive Selection of CD8$^+$ T Cells in the Thymus

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## Summary

**Mice deficient in the gene encoding the peptide transporter associated with antigen processing (*TAP1*) have drastically reduced levels of surface expression of MHC class I molecules and few CD8$^+$ T cells. Addition of class I binding peptides to cultured fetal thymi from TAP1 mutant mice invariably allowed the rescue of class I expression, while only some of these peptides promoted the positive selection of CD8$^+$ T cells, which were polyclonal and specific for the peptide–MHC complex. A nonselecting peptide was converted to a mixture of selecting peptides when the residues involved in the interaction with TCRs were altered. A mixture of self-peptides derived from C57BL/6 thymi induced positive selection of CD8$^+$ T cells at concentrations that gave relatively low class I surface expression. The implication of these observations is that self-peptides determine, in part, the repertoire of specificities exhibited by CD8$^+$ T cells.**

## Introduction

Two types of cellular selection take place in the thymus, both of which are central to the repertoire determination of T cells. First, some autoreactive T cells are eliminated in the thymus by a process referred to as negative selection (for a review see Schwartz, 1989). Second, through positive selection (Bevan, 1977; Zinkernagel et al., 1978), the thymus ensures that T cells leaving the thymus are capable of major histocompatibility complex (MHC)–restricted recognition of antigen; that is, they possess the capacity to recognize antigen presented by products of the self-MHC (Zinkernagel and Doherty, 1974; Fink and Bevan, 1978). Positive selection is thought to involve engagement of T cell receptors (TCRs) on immature CD3$^{lo+}$CD8$^+$CD4$^+$ thymocytes with self-MHC products expressed on the surface of thymic epithelial cells (Lo and Sprent, 1986): only those thymocytes expressing TCRs with sufficient affinity for self-MHC products are given the signal for maturation and proceed to leave the thymus and seed the peripheral lymphoid organs (Kisielow et al., 1988a; Sha et al., 1988). Successful engagement of TCRs with MHC class I molecules leads to differentiation into CD8$^+$CD4$^-$ cytotoxic T cells, and engagement with MHC class II molecules leads to differentiation into CD4$^+$CD8$^-$ T helper cells (Teh et al., 1988).

Mature CD8$^+$ T cells recognize peptide antigens presented by self-MHC class I molecules (Townsend et al., 1986). Most of these peptides are generated in the cytosol and transported into the endoplasmic reticulum, where they associate with newly synthesized class I molecules (Morrison et al., 1986; Nuchtern et al., 1989). Experiments with cell lines (Townsend et al., 1989; Schumacher et al., 1990) and mutant mice (Van Kaer et al., 1992), which are defective in the peptide loading of class I molecules, have demonstrated that peptide is a component essential for the assembly, intracellular transport, and stable surface expression of class I molecules. Given this body of evidence, it is almost certain that the class I molecules of thymic epithelial cells seen by the TCRs of immature thymocytes contain peptides. However, it is not known if the peptide is recognized by the TCR, directly or indirectly, during positive selection and thereby contributes to the shaping of the repertoire of selected T cells. In another model of positive selection, peptides shape the T cell repertoire but only TCR–MHC interactions are required. Certain MHC-bound peptides can be ignored and allow these TCR–MHC interactions, while others sterically prevent the appropriate TCR–MHC interactions. In this model, the effects of peptide are indirect. Yet another model is that the role of the peptide is limited to the stabilization of class I expression and has no bearing on repertoire determination. Experiments carried out using MHC mutant mice have produced results that are consistent with the models in which peptides can influence T cell repertoire (Nikolić-Žugić and Bevan, 1990; Jacobs et al., 1990; Sha et al., 1990). However, it is yet to be established that self-peptides contribute to the determination of T cell repertoire.

In this paper, we addressed this issue more directly by exploiting TAP1 (transporter associated with antigen processing 1) mutant mice that were produced by the embryonic stem cell gene targeting technique (Van Kaer et al., 1992). *TAP1* as well as *TAP2* gene products are thought to be essential components of a peptide pump that mediates the entry of peptides from the cytosol into the endoplasmic reticulum. In TAP1 mutant mice, this major route of peptide loading of class I molecules is blocked, and consequently cell surface expression of class I molecules is severely hampered (Townsend et al., 1989; Ljunggren et al., 1990; Attaya et al., 1992). However, as is the case with the TAP2 mutant cell line RMA-S (Ljunggren et al., 1990), some "empty," ill-folded, and heat-unstable class I molecules manage to appear on the surface of cells in TAP1 mutant mice (Van Kaer et al., 1992). These empty class I molecules can be loaded and stabilized in situ with appropriate peptides that are provided extracellularly in the presence of β2-microglobulin (Van Kaer et al., 1992).

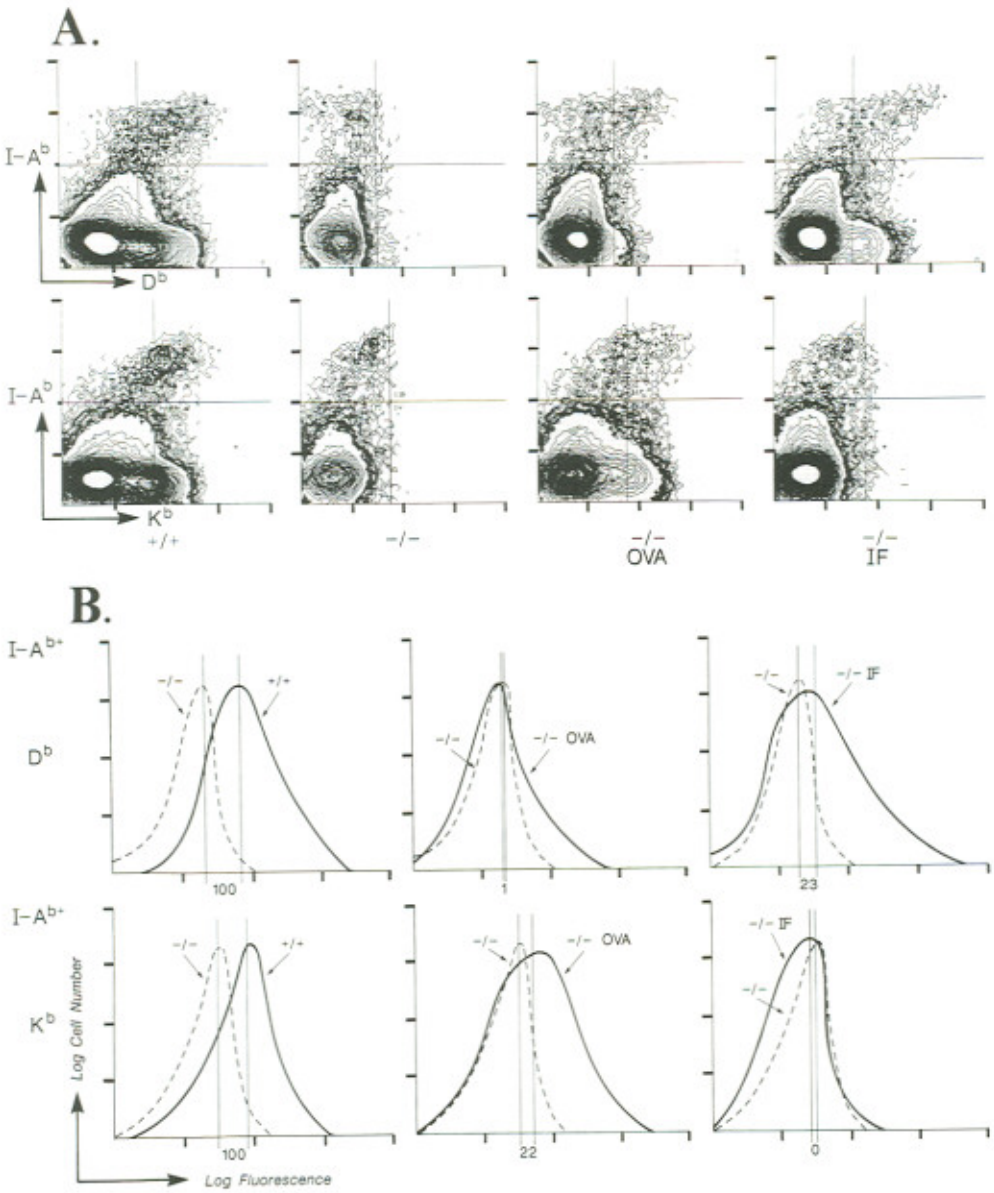



Figure 1. Peptide Stabilizes Class I Surface Expression in TAP1⁻ FTOC

C57BL/6 (+/+) and TAP1⁻ (−/−) thymic lobes were cultured for 10 days in serum-free medium containing 30 μg/ml human β2-microglobulin and where indicated synthetic peptide OVA or IF at a concentration of 500 μM. Cells from protease-digested thymi were stained for I-$A^b$, $K^b$ (AF6.44, α2 specific), and $D^b$ (B22.249R1, α1 specific) surface expression.

(A) Thymic stroma cells ($2 \times 10^5$) pooled from ten protease-digested thymi were stained with anti-I-$A^b$-phycoerythrin MAb and either anti-$K^b$-biotin (then streptavidin–allophycocyanin) or anti-$D^b$-fluorescein isothiocyanate and analyzed by fluorescence-activated cell sorting.

(B) To quantitate the surface expression of class I molecules on the gated I-$A^{b+}$ cells, the log relative fluorescence intensity resulting from staining with labeled class I–specific MAbs was plotted against the log relative cell number. The histogram for TAP1⁻ (−/−) thymi cultured without peptide was compared with the histograms for TAP1⁻ (−/−) thymi cultured with peptide or histograms for TAP1⁺ (+/+) thymi. The differences between the levels of log mean fluorescence intensity are shown and are expressed as a percentage of the TAP1⁺ (+/+) value.

This opened the possibility of using fetal thymic organ culture (FTOC) (Jenkinson et al., 1981) to manipulate class I expression in TAP1 mutant thymi in order to address the question of the role of peptide in positive selection. We have found the following results. First, among a group of synthetic peptides capable of rescuing class I expression on the surface of thymic epithelial cells, only some are capable of inducing positive selection of CD8⁺ T cells. Second, a nonselecting peptide can be converted to a selecting peptide mixture by alterations of some amino acid residues. Third, complex peptide mixtures extracted from thymi are very effective positive selectors. We interpret these results as meaning that peptides do not just stabilize class I molecules on the thymic epithelial cells, but contribute to the specificity of the TCR–MHC interaction during positive selection.

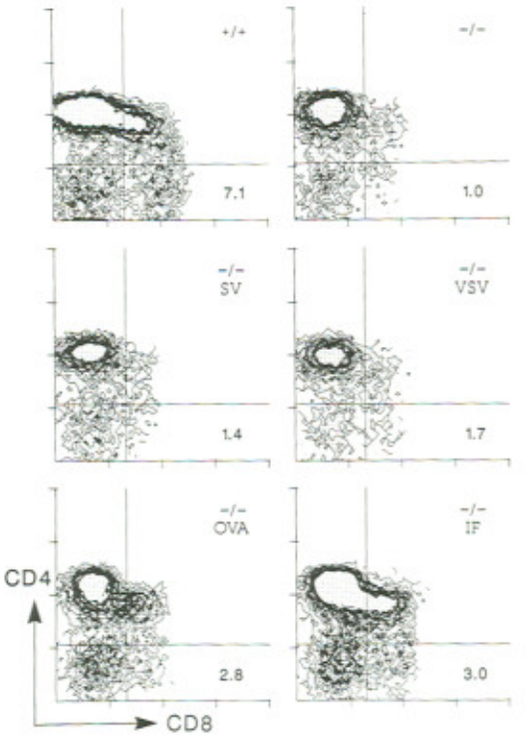



Figure 2. Synthetic Peptides Induce Positive Selection of CD8⁺ Cells in TAP1⁻ FTOC

C57BL/6 (+/+) and TAP1⁻ (−/−) thymic lobes were cultured, as described in Figure 1, with peptide (500 μM) where indicated. The peptides used were as follows: SV, VSV, OVA, and IF. Thymocytes prepared from individual thymic lobes (approximately 1 × $10^6$) by mechanical disaggregation were stained for CD3, CD4, and CD8 surface markers and analyzed by fluorescence-activated cell sorting. Cells (0.5 × $10^5$ to 1.0 × $10^5$) were gated for high expression of CD3 (typically 10%–20% of thymocytes), and then were displayed for log fluorescence intensity resulting from staining with CD4 and CD8. The percentage of these gated cells that were $CD8^+CD4^-$ is indicated.

## Results

### Rescue of MHC Class I Expression on the Cells of Cultured TAP1 Mutant Thymi

Our previous study demonstrated that expression of class I molecules on the surface of splenocytes from TAP1 mutant mice is greatly enhanced by in vitro treatment with class I binding peptides (Van Kaer et al., 1992). We wanted to confirm that such enhancement of class I expression also occurred in cultured fetal thymi. We cultured day 16 fetal thymi lobes from TAP1 mutant mice in the presence or absence of various synthetic peptides (500 μM) and purified human β2-microglobulin (30 μg/ml) (hereafter referred to as TAP1⁻ FTOC). After 10 days, we disaggregated the lobes with protease digestion and measured the degree of surface expression of class I molecules by flow cytometry using appropriate monoclonal antibodies (MAbs).In this experiment, we doubly labeled cells with class I and class II MAbs to allow distinction of class II–positive and class II–negative subpopulations. The former contains cortical epithelial cells thought to play a key role during positive selection by presenting self-MHC molecules to immature thymocytes (Lo and Sprent, 1986).

As expected, expression of class I molecules on the surface of both $I\text{-}A^{b+}$ and $I\text{-}A^{b-}$ cells from TAP1⁻ FTOC was drastically reduced compared with similarly produced TAP1⁺ FTOC (Figure 1). The influenza virus nucleoprotein (NP) peptide (amino acids 366–374) (IF peptide) is recognized by $D^b$-restricted cytotoxic T lymphocytes (Townsend et al., 1986), and the ovalbumin peptide (amino acids 257–264) (OVA peptide) is recognized by $K^b$-restricted cytotoxic T lymphocytes (Carbone and Bevan, 1989). As shown in Figure 1A, TAP1⁻ FTOC in the presence of each of these peptides resulted in the rescue of cell surface expression of the corresponding class I molecule(s) (i.e., $D^b$ or $K^b$) in both $I\text{-}A^{b+}$ and $I\text{-}A^{b-}$ subpopulations (Figure 1A). We quanti-

Table 1. Numbers of Various Types of Thymoctyes in FTOCs

| Experiment Number | Thymus | Peptide | Number of Cells per Thymic Lobe × $10^{-3}$ | | |
|---|---|---|---|---|---|
| | | | $CD3^{b+}$ | | |
| | | | $CD4^+CD8^-$ | $CD4^-CD8^+$ | $CD4^+CD8^+$ |
| 1 | +/+ | – | 5.20 ± 0.67 | 0.78 ± 0.05 | 85.85 ± 1.37 (n = 6) |
| | −/− | – | 6.09 ± 0.97 | 0.19 ± 0.04 | 89.36 ± 0.98 (n = 6) |
| | −/− | OVA | 6.14 ± 0.58 (1%) | 0.42 ± 0.10 (121%) | 81.29 ± 1.47 (n = 7) |
| | −/− | IF | 6.46 ± 0.79 (6%) | 0.53 ± 0.18 (190%) | 85.43 ± 2.23 (n = 6) |
| 2 | +/+ | – | 4.24 ± 0.34 | 0.73 ± 0.06 | 85.24 ± 1.19 (n = 10) |
| | −/− | – | 3.76 ± 1.25 | 0.20 ± 0.06 | 87.45 ± 1.87 (n = 5) |
| | −/− | SV | 3.54 ± 0.41 (6%) | 0.20 ± 0.02 (0%) | 88.39 ± 0.96 (n = 6) |
| | −/− | VSV | 3.50 ± 0.61 (7%) | 0.19 ± 0.05 (5%) | 88.41 ± 1.04 (n = 6) |
| 3 | +/+ | – | 10.33 ± 1.53 | 1.08 ± 0.18 | 88.47 ± 3.9 (n = 4) |
| | −/− | – | 11.71 ± 0.95 | 0.21 ± 0.07 | 106.00 ± 19.28 (n = 4) |
| | −/− | TSP | 10.30 ± 1.64 (12%) | 0.62 ± 0.19 (195%) | 100.52 ± 11.81 (n = 4) |

Absolute cell numbers of thymocytes from C57BL/6 (+/+) or TAP1⁻ (−/−) incubated with or without synthetic peptide (SV, VSV, IF, or OVA [500 μM]) or thymic self-peptides (TSP [250 μM]). The data in experiments 1 and 2 are further described in Figure 3B. The cell numbers are mean values (± SEM) from the analysis of independently cultured thymic lobes (n = number of lobes analyzed). The numbers in parentheses indicate proportional increments of the corresponding types of cells in TAP1⁻ FTOC upon treatment with the indicated peptide or peptide mixture. Indicated is the number of stained cells per thymic lobe. The total number of cells per thymic lobe was about 5 × $10^5$.

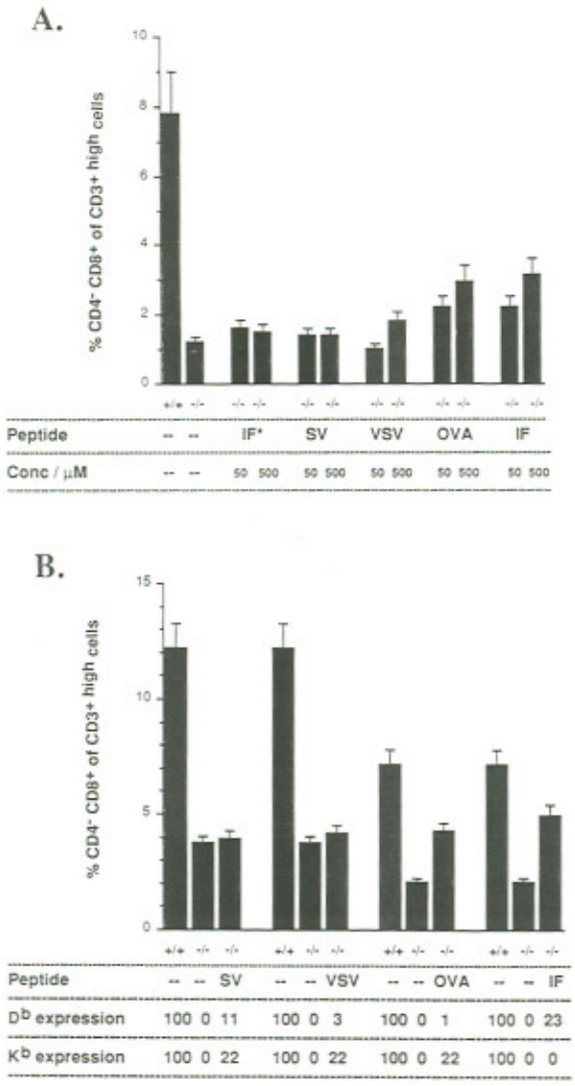



Figure 3. Some but Not All Peptides That Bind Class I Molecules Induce Positive Selection of CD8⁺ Cells

(A) C57BL/6 (+/+) and TAP1⁻ (−/−) thymic lobes were cultured, as described in Figure 1, with peptide (50 or 500 μM) where indicated. Peptide IF* is specific for the Kᵈ class molecule and does not bind any H-2ᵇ class I molecule (Falk et al., 1991; P. G. A.-R., unpublished data). After 10 days of incubation, thymocyte suspensions were prepared by mechanical disaggregation, cells were stained as in Figure 2, and then the percentage of CD8⁺CD4⁻ cells of the CD3ʰⁱ⁺ population was determined. The levels indicated are based on the analysis of four separately cultured thymic lobes (mean ± SEM). Addition of either IF* or SV (both concentrations) did not result in significant changes in the levels of CD3ʰⁱ⁺CD4⁻CD8⁺ cells ($P > 5\%$). Addition of VSV, only at the higher concentration, resulted in a significant increase ($0.5\% < P < 5\%$), whereas at both concentrations OVA and IF had a significant effect ($0.5\% < P < 5\%$). Similar results were obtained in two other independently performed experiments.

(B) C57BL/6 (+/+) and TAP1⁻ (−/−) thymi were cultured for 7 days with peptide (500 μM) where indicated. The class I specificities for the synthetic peptides used were as follows: Kᵇ/Dᵇ for SV (Schumacher et al., 1990; Van Kaer et al., 1992); Kᵇ for VSV (van Bleek and Nathenson, 1990; Van Kaer et al., 1992); Kᵇ/Dᵇ for OVA (Carbone and Bevan, 1989; Van Kaer et al., 1992); Dᵇ for IF (Townsend et al., 1986; Van Kaer et al., 1992). The levels for the percentage CD8⁺CD4⁻ cells of the CD3ʰⁱ⁺ population were based on data obtained from the analysis of between five and ten separately cultured thymi (mean ± SEM). Addition of either SV or VSV did not result in a significant change in the levels of CD3ʰⁱ⁺CD4⁻CD8⁺ cells compared with −/− thymi alone ($P > 5\%$), whereas the addition of OVA ($0.5\% < P < 5\%$) or IF ($0.05\% < P < 0.5\%$) resulted in significant increases. The levels of Dᵇ and Kᵇ expression were determined on the I-Aᵇ⁺ stromal cells obtained from the same thymi that were analyzed for CD3, CD4, and CD8 surface markers. The levels of surface class I expression were determined as described in Figure 1 and are expressed as a percentage of the TAP1⁺ (+/+) control level less the background level.

tated the level of Dᵇ and Kᵇ expression on gated I-Aᵇ⁺ cells by determining the mean levels of fluorescence staining intensity with class I–specific MAbs (Figure 1B). The stabilization pattern observed with the cells in FTOC was very similar to that of TAP1-deficient splenocytes (Van Kaer et al., 1992) or RMA-S cells (Townsend et al., 1989; Schumacher et al., 1990; Deres et al., 1992) treated with the same peptides in vitro.

### Some but Not All Peptides That Stabilize Class I Expression in the Thymus Induce Positive Selection of CD8⁺ T Cells

Our previous study demonstrated that the positive selection of CD8⁺ T cells, but not that of CD4⁺ T cells, is severely hampered in the thymus of the TAP1 mutant mice. The data shown in Figure 2 and Table 1 confirm that such a CD8⁺ T cell–specific deficiency in positive selection also occurs in the TAP1⁻ FTOC. In 10-day-old TAP1⁺ FTOC, about 7% of CD3ʰⁱ-positive (CD3ʰⁱ⁺) thymocytes were CD8⁺CD4⁻ (hereafter, referred to as CD8⁺ cells), while the proportion of this subpopulation was only 1% in similarly cultured TAP1⁻ FTOC. The proportional decrease of the CD8⁺CD4⁻ thymocytes in the TAP1⁻ FTOC reflects a true reduction in the number of these thymocytes per thymic lobe rather than an increase in the number of other thymocyte subpopulations, such as CD4⁺CD8⁺ cells or CD3ʰⁱ⁺CD4⁺CD8⁻ cells (hereafter, referred to as CD4⁺ cells) (Table 1). The number of cells belonging to the latter subpopulations did not differ significantly in TAP1⁻ FTOC and TAP1⁺ FTOC.

Having demonstrated that class I surface expression can be partially restored by exogenously provided peptides in TAP1⁻ FTOC (see above), we wanted to find out whether the production of CD8⁺ cells is also restored in these organ cultures by the peptide treatment. Our data indicate that CD8⁺ cell levels are raised by some but not all peptides tested. The effect of peptides is specific in that the levels of CD4⁺ or CD4⁺CD8⁺ cells are unaltered (Table 1). As shown in Figure 3A, the addition of Sendai virus (SV) NP peptide (324–332) (hereafter, referred to as SV peptide) to TAP1⁻ FTOC had no effect on the level of CD8⁺ cells when compared with TAP1⁻ FTOC without a peptide or with a peptide that does not bind H-2ᵇ class I molecules, such as the influenza NP (amino acids 147–154) peptide (IF* peptide) (Falk et al., 1991). The vesicular stomatitis virus (VSV) NP peptide (52–59) (VSV peptide) had a variable effect on CD8⁺ cell levels, either increasing them slightly above background or not at all. In contrast, either the OVA peptide or IF peptide clearly and consis-

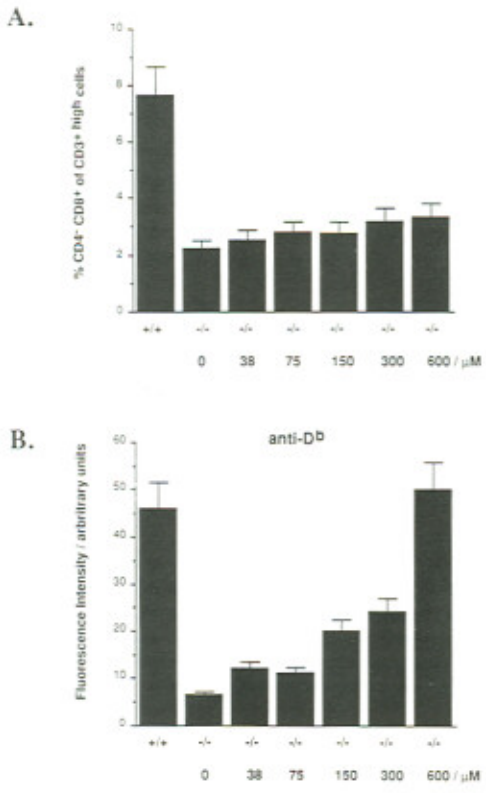



Figure 4. The IF Peptide Stabilizes $D^b$ in the Thymus and Induces Positive Selection of $CD8^+$ Cells

C57BL/6 (+/+) and $TAP1^-$ (−/−) thymic lobes were cultured as before with the IF peptide at the concentrations shown for 10 days. Thymocyte suspensions were prepared from individual thymic lobes by mechanical disaggregation, stained for CD3, CD4, and CD8 surface markers and $I\text{-}A^b$, $K^b$, and $D^b$ surface expression, and then analyzed by fluorescence-activated cell sorting.

(A) The percentage of $CD4^-CD8^+$ cells of the $CD3^{hi+}$ population was determined as before; the levels indicated are the mean values (± SEM) of determinations done on four separate thymic lobes. The correlation between peptide concentration and level of $CD3^{hi+}CD4^-CD8^+$ cells was significant (F test [$p = 0.0158$], correlation coefficient [$R = 0.945$, $R2 = 0.80$], t test [$p = 0.016$]).

(B) The levels of $D^b$ and $K^b$ expression were determined on the nonadherent, dendritic cell population ($I\text{-}A^{b+}$) as in Figure 1. There was no detectable rescue of $K^b$ surface expression by the IF peptide.

tently increased the level of $CD8^+$ cells above background levels. In another set of experiments, the results of which are shown in Figure 3B, we measured the levels of $CD8^+$ thymocytes as well as class I surface expression in the $I\text{-}A^{b+}$ cell subpopulation derived from the same FTOCs. Each of the four peptides tested restored class I surface expression on the $I\text{-}A^{b+}$ cells with the expected specificity (Schumacher et al., 1990; van Bleek and Nathenson, 1990), but as shown in Figure 3A, only the OVA and IF peptides were good $CD8^+$ cell selectors, the VSV peptide was a poor selector, and the SV peptide was apparently a nonselector. That the restoration of $CD8^+$ cell generation and surface stabilization of class I molecule expression are both dependent on the concentration of added peptide is illustrated in Figure 4 for the IF peptide.

In normal thymic development different class I MHC molecules (D or K) select discrete $CD8^+$ T cell subpopulations (Sha et al., 1990; Nikolić-Žugić and Bevan, 1990). We wanted to see whether this was the case in our $TAP1^-$ FTOC. To this end, we added two peptides simultaneously and then compared the levels of selected $CD8^+$ cells in these cultures with the levels observed for those cultures in which the peptides were added separately. We found that for the pairs of peptides OVA (primarily $K^b$ binding) and IF ($D^b$ binding), and VSV (primarily $K^b$ binding) and IF, the levels of selected $CD8^+$ cells in cultures incubated with both peptides were approximately the sum of the levels observed in cultures with the peptides added separately (Figure 5). For the SV and VSV peptide pair no significant selection of $CD8^+$ cells was observed. This indicates that in the $TAP1^-$ FTOC system each peptide–MHC combination (such as IF-$D^b$ or OVA-$K^b$) selected a discrete $CD8^+$ T cell population, therefore implying that the positive selection is MHC allele specific.

We conclude that the ability of a peptide to drive positive selection of class I–restricted T cells is not solely based on its ability to stabilize MHC class I molecule expression on the surface of selecting cells.

### The Nonselecting SV Peptide Can Be Converted to a Selecting Peptide Mixture by Amino Acid Replacements

The specificity models predict that, in general, the more diverse the selecting peptides added to $TAP1^-$ FTOC, the more T cell clones selected and hence the greater the number of $CD8^+$ T cells in the culture. To test this hypothe-

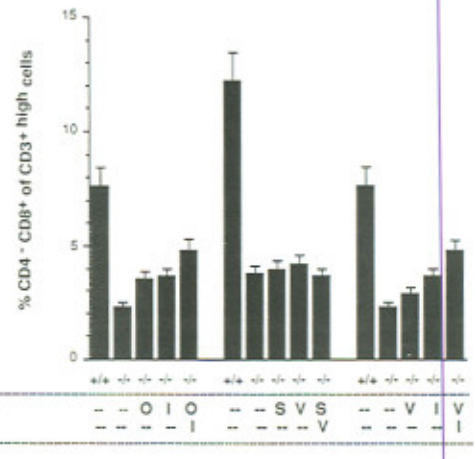



Figure 5. Unique MHC–Peptide Complexes Select Discrete Populations of $CD8^+$ Cells

C57BL/6 (+/+) and $TAP1^-$ (−/−) thymic lobes were incubated for 9 days with peptide(s), where indicated, at a concentration of 500 μM each. OVA is indicated by O, IF by I, SV by S, and VSV by V. Thymocytes were analyzed for CD3, CD4, and CD8 markers, and the percentages of $CD8^+CD4^-$ cells of the $CD3^{hi+}$ population were determined as before. The levels indicated are the mean values (± SEM) of determinations done on four separate thymic lobes. Addition of either OVA, IF, or OVA plus IF resulted in significant increases in the levels of $CD3^{hi+}CD4^-CD8^+$ cells compared with −/− thymi alone ($0.5\% < P < 5\%$), whereas addition of SV, VSV, or SV plus VSV did not ($P > 5\%$). Addition of VSV did not result in a significant change ($P > 5\%$); however, addition of IF or VSV plus IF did ($0.05\% < P < 0.5\%$).

| Residue No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| SV-NP Peptide | F | A | P | G | N | Y | P | A | L |
| Variant Residues | K | R | G<br>D | Y<br>F | V | | Q<br>K | G<br>S<br>T | |

Figure 6. A Complex Mixture of Peptides Based on the SV Sequence

The anchor residues at positions 6 (tyrosine) and 9 (leucine) are present in every peptide in the mixture (SV-M). All other amino acid positions were varied between either 2, 3, or 4 different residues.

sis, we synthesized a mixture of 864 different 9-mer peptides in which the two known class I anchor residues of the SV peptide, tyrosine and leucine at positions 6 and 9, respectively, were retained but the other residues were altered, as shown in Figure 6. When this peptide mixture was added to TAP1$^-$ FTOC, $K^b$ expression, and to a lesser extent $D^b$ expression, was increased on the surface of thymic epithelium (Figure 8). In contrast with the parental SV peptide, the peptide mixture could promote the generation of CD8$^+$ cells to a great extent (Figures 7 and 8).

These results suggest that each class I binding peptide can drive the positive selection of a distinct, albeit overlapping, subset of T cell clones, supporting the specificity models.

### A Mixture of Peptides Extracted from the Thymus Is an Effective Selector

We prepared a crude mixture of peptides from the thymi of C57BL/6 mice by acid extraction and partial purification by ultrafiltration (Udaka et al., 1992). The minimum complexity of the peptide preparation was estimated by high pressure liquid chromatography to be 1000. When this mixture of self-peptides was added to TAP1$^-$ FTOC, a substantial increase in the level of selected CD8$^+$ T cells compared with TAP1$^-$ FTOC without peptide or with the SV peptide was observed (Figure 8). This occurred despite the fact that the level of class I expression on the surface of I-$A^{b+}$ thymic cells was relatively low (Figure 8). These results suggested that the mixture contained self-peptides that were effective in selecting relatively large numbers of different T cell clones.

### Selected CD8$^+$ T Cells Are Polyclonal

To assess the diversity of CD8$^+$ T cells selected by peptides, we determined the relative proportion of these cells expressing different TCR Vβ regions. As shown in Figure 9, for each CD8$^+$ thymocyte population selected by a single peptide or by the mixture of self-peptides, we observed the presence of all of the Vβ$^+$ subpopulations tested, although the relative proportions of some Vβ$^+$ subpopulations varied with the selecting peptide and differed from that of the TAP1$^+$ control culture.

### Discussion

In this study we examined the effect of various peptides, added to TAP1$^-$ FTOCs, on surface expression of class I molecules on thymic epithelial cells as well as on the

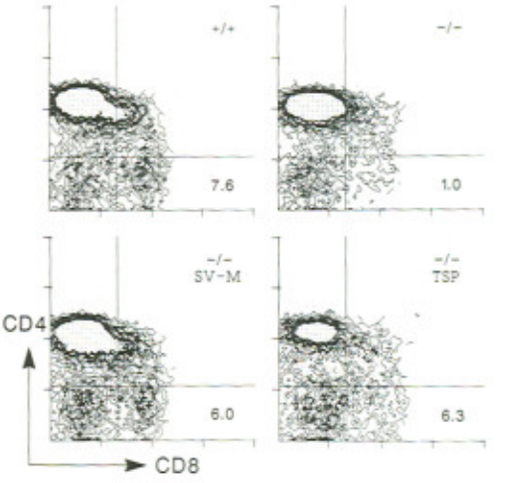
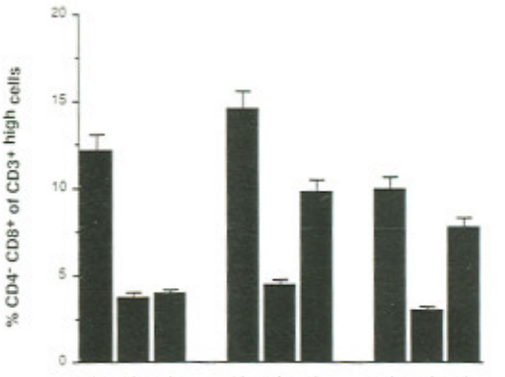



Figure 7. Complex Mixtures of Peptide Induce Positive Selection of CD8$^+$ Cells in TAP1$^-$ FTOC

C57BL/6 (+/+) and TAP1$^-$ (−/−) thymic lobes were cultured, as described in Figure 1, with synthetic peptide mixture (SV-M) (500 μM) or thymic self-peptides (TSP) (250 μM) where indicated. Thymocyte suspensions were prepared by mechanical disaggregation of individual thymic lobes, and the percentage of CD8$^+$CD4$^-$ cells of the CD3$^{hi+}$ population was determined as in Figure 2.




| Peptide | -- | -- | SV | -- | -- | SV-M | -- | -- | TSP |
|---|---|---|---|---|---|---|---|---|---|
| Complexity | -- | -- | 1 | -- | -- | 864 | -- | -- | >1000 |
| $D^b$ expression | 100 | 0 | 11 | 100 | 0 | 1 | 100 | 0 | 3 |
| $K^b$ expression | 100 | 0 | 22 | 100 | 0 | 13 | 100 | 0 | 0 |

Figure 8. Complex Mixture of Peptides Induces the Positive Selection of CD8$^+$ Cells

C57BL/6 (+/+) and TAP1$^-$ (−/−) thymi were cultured for 7 days with synthetic peptide (SV or SV-M) (500 μM) or thymic self-peptides (TSP) (250 μM) where indicated. The levels for the percentages of CD8$^+$CD4$^-$ cells of the CD3$^{hi+}$ population were based on data obtained from the analysis of between five and ten separately cultured thymi (mean ± SEM). Similar results were obtained in two independently performed experiments. The levels of $D^b$ and $K^b$ expression were determined on the I-$A^{b+}$ stromal cells obtained from the same thymi that were analyzed for CD3, CD4, and CD8 surface markers. The levels of surface class I expression were determined as in Figure 1 and are a percentage of the normal control less the background level.

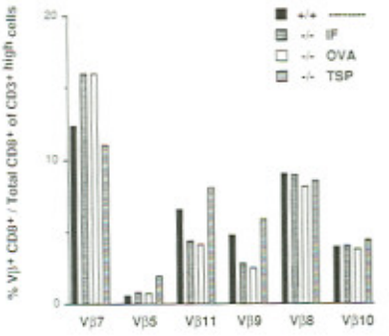



Figure 9. Single Peptides Induce the Positive Selection of Polyclonal CD8+ Populations

C57BL/6 (+/+) and TAP1⁻ (−/−) thymic lobes were cultured for 7 days with synthetic peptide (500 μM) or thymic self-peptides (TSP) (250 μM). In each case the percentage of Vβ+ CD8+ per total CD8+ of the $CD3^{hi+}$ population was determined by staining and fluorescence-activated cell sorting analysis of thymocyte suspensions prepared from between five and ten pooled thymi.

generation of CD8+ T cells. According to the stability model, the extent of the stabilization of class I expression on the surface of $I\text{-}A^{b+}$ thymic cells should correlate, within a limit, to the extent of the generation of CD8+ thymocytes. However, our data demonstrate that in three separate instances these two events do not correlate. First, the single peptide experiment showed that all four peptides tested restored class I expression to a similar extent, and yet some (IF and OVA) were clearly more effective in inducing CD8+ cell generation than others (SV and VSV). Second, a mixture of peptides synthesized based on the sequence of the SV peptide restored class I expression to an extent similar to the original SV peptide. However, the mixture was a much more effective CD8+ T cell selector than the SV peptide. Finally, the self-peptide mixture was a relatively poor stabilizer of class I molecules and yet was very effective at inducing CD8+ cell generation. Thus, we conclude that the peptide's role during positive selection is more than mere stabilization of class I expression on the surface of thymic epithelial cells.

We assume that the abundance of CD8+ T cells generated in FTOC primarily reflects the diversity of T cell clones rather than proliferation of preexisting CD8+ cells. This is supported by the observation that virtually all of the CD8+ cells present in TAP1+ FTOC, or peptide-treated or untreated TAP1⁻ FTOC, are small thymocytes (our unpublished data). The straightforward interpretation of the results obtained with the SV peptide and the synthetic peptide mixture is that the peptide diversity is instrumental in the positive selection of a large number of T cell clones. Thus, in the FTOC supplemented with the single SV peptide, class I (both $K^b$ and $D^b$) expression on the surface of thymic epithelial cells is restored to a substantial level, but there is no diversity in the bound peptide. Consequently, only a very limited number of T cell clones, not enough to be detected above the background level, interact with the peptide–class I complexes with appropriate avidity and are induced to mature to CD8+ cells. In the FTOC with the synthetic mixture of peptides, class I restoration is no higher compared with the FTOC with the SV peptide alone, but the peptides presented are diverse. We estimate that about 20% of the 864 peptides added to the culture, namely, about 170 peptides, bind to and stabilize $K^b$ and $D^b$ molecules (our unpublished data). Each of these bound peptides may be capable of inducing the maturation of a few T cell clones like the SV peptide, but cumulatively, the peptide mixture induces the maturation of a sizable number of T cell clones.

The role of peptides in shaping T cell repertoire may be indirect, at least in part. Our data are consistent with the notion that steric hindrance of TCR–MHC interactions by some but not other peptides would disallow positive selection. The more buried and less accessible the peptide, the better positive selector such a class I–peptide complex would be.

Considerations similar to the ones presented above also apply to the result of the FTOC supplemented by self-peptides extracted from the thymus. In this case, it is likely, if not certain, that the complexity of the class I binding peptides is considerably greater than the ~170 peptides in the synthetic peptide mixture. Our results suggest that the natural peptide mixture is capable of inducing the maturation of a large number of T cell clones even at a concentration that gives relatively low surface class I restoration. The thymic peptide mixture may be relatively rich in those peptides that have a capacity to cross-react with a relatively large number of T cell clones and induce their maturation. The low surface expression of class I molecules can be explained by the low concentration of class I binding peptides present in the mixture.

The above considerations are consistent with specificity models of positive selection. We cannot formally rule out the possibility, however, that the high degree of positive selection observed in the FTOC with the synthetic or natural peptide mixture is mediated by one or a few superpeptides that have the capacity to promote the maturation of all T cell clones to be restricted by either $K^b$ or $D^b$ molecules. Nonetheless, we consider this possibility highly unlikely because it is difficult to imagine how a single peptide could effectively interact with such a diverse set of TCRs in order to initiate the necessary signal transduction.

In the FTOC supplemented with a foreign single peptide, it is unlikely that the CD8+ cells selected include T cell clones that would react with the same peptide in a peripheral response. For instance, the IF peptide, when injected into mice bearing an IF-specific transgenic T cell clone, caused negative selection of the T cell clone (Mamalaki et al., 1992). Therefore, it seems likely that these foreign peptides induced positive selection by cross-reacting with a relatively large number of T cell clones, as supported by the complexity of Vβ segment usage by the selected cells. It is also likely that the cross-reaction was artificially pronounced by the relatively high density of undiversified peptide–class I complexes on the surface of thymic epithelial cells. Selection of T cell clones with low affinity TCRs is likely to have occurred through the increase in avidity provided by these large numbers of homogeneous class I–peptide complexes. It is interesting that the SV peptide, even when present on the surface of thymic epithelium at

relatively high density, did not result in a detectable level of $CD8^+$ cell generation. The structure of $K^b$ crystallized with SV and VSV peptides has been determined by X-ray crystallography (Fremont et al., 1992). The structure reveals that the SV peptide adopts a conformation distinct from that of the VSV peptide complex. This feature of the SV peptide may have prevented a broad cross-reaction with TCRs of immature thymocytes.

The possibility that the different effects of the single class I binding peptides on the induction of $CD8^+$ cell levels were due to their differential effect on negative selection, such that the OVA and IF peptides were poor negative selectors and VSV and SV peptides were good negative selectors, is unlikely. This is because the absolute number of $CD4^+CD8^+$ and $CD4^+CD8^-$ cells did not vary significantly in the FTOCs supplemented with the various peptides (Table 1). If negative selection, rather than positive selection, was the major factor in determining the levels of $CD8^+$ cells, one would have expected corresponding variations in the levels of $CD4^+CD8^+$ and $CD4^+CD8^-$ cells (Kisielow et al., 1988b; Pircher et al., 1989).

Positive selection ensures that T cells leaving the thymus are capable of recognizing antigen presented by products of self-MHC. To preserve the remarkable ability of the immune system to respond to a diverse and rapidly changing set of antigens, one might argue that it is not beneficial to let self-peptides determine the specificity of positive selection. This is because peptides derived from self-proteins are probably less diverse than those derived from the microbial pathogens as a whole that may confront the immune system. Furthermore, the ability of self-proteins (and so self-peptides) to generate genetic variants is severely limited compared with that of microbial proteins, because the generation time of a vertebrate host is far greater than that of a microbial pathogen. However, our results indicate that peptide does indeed contribute to the specificity of positive selection, thus supporting the specificity model. One might imagine that it is inevitable that, given the structure of the class I MHC–peptide complex, the specificity of TCR recognition be influenced by the resident peptide. Perhaps the answer to the apparent dilemma lies in the possibility that the specificity of the interaction of the TCR with MHC–peptide complexes during positive selection is relatively broad.

## Experimental Procedures

### Mice

Control C57BL/6 mice (obtained from The Jackson Laboratory, Bar Harbor, ME) were maintained and bred under standard conditions. TAP1-deficient mice ([129/SvJ × C57BL/6]F2; Van Kaer et al., 1992) were maintained and bred in autoclaved cages with autoclaved food and water. Pregnancies were timed from the first day of plug observation.

### Thymic Organ Cultures

Fetal thymi were cultured according to procedures described previously (Jenkinson et al., 1981). In brief, whole thymi or thymic lobes were removed from day 16 fetuses and placed on nitrocellulose filters (Millipore, 0.2μ pore size) supported by collagen sponges (Colla-Tec) saturated in RPMI–1% Nutridoma Media Supplement (Boehringer Mannheim), $5 \times 10^{-5}$ M β-mercaptoethanol, glutamine, nonessential amino acids, sodium pyruvate, penicillin, and streptomycin. The medium was then supplemented with human β2-microglobulin (99% pure, Cortex Biotec), and synthetic or natural peptides were added where indicated. The cultures were fed every other day by complete medium replacement with fresh peptide, where indicated.

### Flow Cytometric Analyses

The following MAbs were used: anti-CD4 (allophycocyanin labeled), anti-CD8a (fluorescein isothiocyanate labeled), anti-CD3ε (R phycoerythrin labeled), anti-$IA^b$ phycoerythrin labeled, anti-$K^b$ fluorescein isothiocyanate labeled (AF6.44 α2 specific), anti-Vβ7 biotin labeled, anti-Vβ5 biotin labeled, anti-Vβ11 biotin labeled, anti-Vβ9 biotin labeled, anti-Vβ8 biotin labeled, anti-Vβ10 biotin labeled (Pharmingen), and anti-$D^b$ biotin labeled (B22-249R1 α1 specific, American Type Culture Collection).

Thymocyte suspensions were prepared from thymic lobes according to standard procedures and incubated in staining buffer (phosphate-buffered saline, 1% fetal calf serum, and 0.1% $NaN_3$) with MAbs for 30 min at 4°C. Cells were then washed and, where appropriate, incubated with streptavidin–allophycocyanin (Pharmingen) for a further 15 min to detect MAb–biotin binding. After washing, all viable cells (usually between $0.5 \times 10^5$ and $1.0 \times 10^5$ cells) were analyzed using a FACStar$^{plus}$ flow cytometer (Becton-Dickinson). Dead cells were excluded from the analysis by staining with propidium iodide and based on their forward and sideways light scattering properties.

Thymic epithelial cell suspensions from five to ten thymi were prepared using a method modified from Farr et al. (1986). After thymocyte suspensions were prepared, the particulate stromal matter was incubated in digestion buffer (phosphate-buffered saline, 2.5% trypsin [Sigma], 384 U/ml collagenase type IV [Sigma]) for 10 min at 37°C with vigorous agitation. Digestion was stopped by adding an equal volume of fetal calf serum. The cells were washed twice and then stained with MAbs.

### Peptides

Amino acid sequences of synthetic peptides are as follows: VSV peptide (amino acids 52–59), RGYVYQGL; SV peptide (amino acids 324–332), FAPGNYPAL; IF peptide (amino acids 366–374), ASNENMETM; IF* peptide (amino acids 147–154), TYQRTRALV; OVA peptide (amino acids 257–264), SIINFEKL. These peptides as well as the SV–NP mix (Schumacher et al., 1992) were synthesized and purified by high pressure liquid chromatography.

The thymic self-peptide mixture was prepared as described by Udaka et al. (1992). In brief, about 200 thymi from 6- to 8-week-old female C57BL/6 mice were homogenized in 0.7% trifluoroacetic acid and then sonicated. The extracts were incubated for 30 min on ice and then clarified by centrifugation at 31,000 × g and subjected to ultrafiltration through a centricon 10 membrane (molecular mass cut off 10 kd; Amicon). Filtrates were dried by Speed Vac and dissolved in 0.1% trifluoroacetic acid. This peptide extract was then further purified by reverse-phase chromatography using a C10 Sep-Pak cartridge (Waters). The concentration of 9 and 8 amino acid long peptides present in thymic self-peptide mixtures was estimated based on absorbance by reverse-phase high pressure liquid chromatography analysis of the extracts in comparison with synthetic peptide mixtures of known composition and concentration.

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