

## **Positive Selection of CD8<sup>+</sup> T Cells Induced by Major Histocompatibility Complex Binding Peptides in Fetal Thymic Organ Culture**

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### **Summary**

We have used an in vitro system to study the effects of major histocompatibility complex class I binding peptides on thymic development. Fetal thymus lobes from mice deficient in the class I light chain ( $\beta_2$  microglobulin or  $\beta_2M^{-/-}$ ) were cultured for 10 d in vitro, during which time T cell precursors develop into mature T cells. In these organ cultures, as in the adult or neonatal  $\beta_2M^{-/-}$  thymus, CD8<sup>+</sup> mature T cells did not develop, demonstrating that the mature T cells seen during early murine thymic development are the result of the positive selection process. To these cultures we added various class I binding peptides with or without a source of exogenous  $\beta_2M$ . CD8<sup>+</sup> T cells developed to various degrees only in the presence of  $\beta_2M$  and peptides. Using peptide mixtures of differing complexity, we showed that the efficiency of this process is dependent more on peptide complexity than on peptide concentration. These data argue for a specific role for peptides in the process of positive selection. Furthermore, this culture system should be useful in identifying peptides that can promote positive selection of cells expressing a specific T cell receptor (TCR) in TCR transgenic mice.

CD8<sup>+</sup> T cells mature in the thymus and are subject to the processes of positive and negative selection during their development (1). The process of positive selection, whereby T cells with the capacity to recognize antigens in the context of the individual MHC molecules are rescued from programmed cell death, is known to require the interaction of the TCR with MHC molecules on thymic stromal cells. It has been proposed that MHC-self-peptide complexes, as opposed to MHC molecules alone, mediate this event. This is quite likely to be the case since very few "empty" class I molecules are found on the cell surface (2). However, whether self-peptides play a specific role in this process or whether they serve only to maintain class I H chains in a particular conformation is not clear.

We sought to develop an in vitro system to test the role of class I binding peptides in CD8<sup>+</sup> T cell development. Fetal thymic organ culture (FTOC) provides a way to study thymic selective events in vitro (3). In FTOC, the immature T cells present in the thymus at gestational age day 16 develop in the undisturbed lobe. Assessment of the phenotype of the cells over time in this system suggests that development in vitro mimics that in vivo, and that functional T cells can be obtained (4). Because class I molecules with self-peptides are present in lobes from normal animals, we have used thymic lobes from mice deficient in  $\beta_2M$  (5, 6). These mice have low surface levels of class I because the H chains alone are

not efficiently transported. As a result, these mice profoundly lack CD8<sup>+</sup> mature T cells. It has been shown previously that despite the low levels of surface class I in APCs lacking  $\beta_2M$ , T cells can recognize them if specific peptide and  $\beta_2M$  are provided exogenously (7). We have therefore provided exogenous class I binding peptides and a source of  $\beta_2M$  and assessed CD8<sup>+</sup> T cell development in FTOC.

### **Materials and Methods**

**Animals.** Time-mated C57BL/6 females were purchased from Simonsen (Gilroy, CA). H-2<sup>b</sup>  $\beta_2M^{-/-}$  mice (5) were obtained from Beverly Kohler (University of North Carolina, Chapel Hill, NC) and maintained in our animal facilities. The day the animals were plugged was considered day 1.

**Peptides.** Octamer (OVA 257-264) or nonamer (influenza nucleoprotein 366-374) class I binding peptides were synthesized on a synthesizer (model 430A; Applied Biosystems Inc., Foster City, CA) at the Howard Hughes Chemical Synthesis facility, University of Washington. Acid extracts of spleen were prepared as described (8). Briefly, C57BL/6 spleens were homogenized and then sonicated in 0.1% TFA. The material was filtered through a 10-kD cutoff membrane (Amicon, Beverly, MA), lyophilized, and resuspended in PBS.

Random peptide mixtures were synthesized in *Escherichia coli* as described using a fusion protein purification strategy (9). A self-priming degenerate oligonucleotide (encoding IEGRSXIXFXXL\*\*\*

flanked by BamHI and PstI sites) was extended, restricted with BamHI and PstI, and cloned into the BamHI and PstI sites of the pMAL-c vector (New England Biolabs Inc., Beverly, MA). Pools of clones were grown to 0.5 OD<sub>600</sub> followed by a 3-h isopropyl  $\beta$ -D-thiogalactoside induction. Fusion proteins were purified by affinity chromatography, and the COOH-terminal octameric peptides released by factor X<sub>2</sub> cleavage after the IEGR site. After passage through a 10-kD cutoff membrane, this material was further purified by HPLC fractionation on a C18 column (Millipore Corp., Milford, MA). Peptide-containing fractions were lyophilized, resuspended in PBS, and pooled. Total protein concentration was determined by the BCA assay (Pierce, Rockford, IL).

**FTOC.** Thymuses from day 16 fetal mice were placed on cellulose ester filters (Millipore Corp.) resting on Gelfoam sponges (Upjohn, Kalamazoo, MI) in a 6-well plate. Sponges and filters were prewet in culture medium (RPMI 1640, 1% Nutridoma SP [Boehringer Mannheim, Indianapolis, IN], 1 mM sodium pyruvate, 0.1 mM nonessential amino acids,  $5 \times 10^{-5}$  M 2-ME, 2 mM L-glutamine, and 100  $\mu$ g/ml each of penicillin and streptomycin) with or without 10% FCS (Hyclone Laboratories, Logan, UT) and peptides. The dishes were kept at 7% CO<sub>2</sub> in a humidity chamber at 37°C for 10 d, during which time the media was replenished every other day. Lobes were harvested by pressing through a steel mesh. Typical cell yields were  $5 \times 10^5$ /lobe.

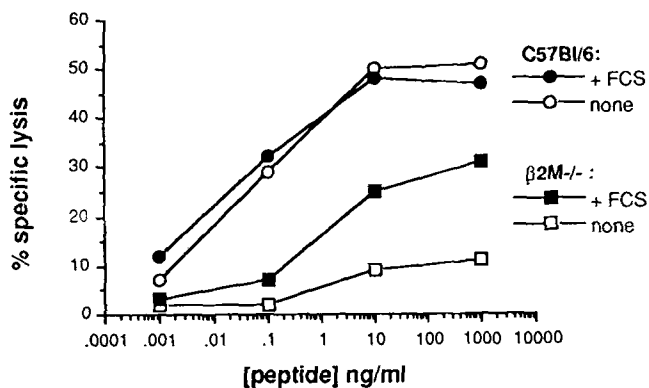
**Flow Cytometry.** Thymocytes from adult mice or FTOC were stained with the following reagents: PE-L3T4, anti CD4; FITC-53.6.7, anti CD8 (both from Becton Dickinson & Co., Mountain View, CA); biotin or FITC H57-597, anti TCR- $\alpha/\beta$  (gift of P. Fink, University of Washington, Seattle, WA); tri-color streptavidin (Caltag Laboratories, San Francisco, CA); Y3, anti K<sup>b</sup>; and 28.14.8, anti D<sup>b</sup> (both from American Type Culture Collection, Rockville, MD) were detected with FITC goat anti-mouse IgG<sub>1</sub>,A (Cappel Laboratories, West Chester, PA). Samples were analyzed using a FACScan<sup>®</sup> (Becton Dickinson & Co.). After gating on live cells by forward and side angle scatter, 100,000 events were collected.

**CTL Assays.** Blast cells were prepared by culturing spleen cells with Con A (2.5  $\mu$ g/ml) and LPS (1  $\mu$ g/ml) for 2 d in AIM-V serum-free medium (Gibco, Grand Island, NY). They were labeled with <sup>51</sup>Cr-sodium chromate and pulsed with OVA 257-264 peptide in the presence or absence of 10% FCS as a source of  $\beta_2$ M before use as targets for the OVA plus H-2K<sup>b</sup> specific CTL clone GA4.

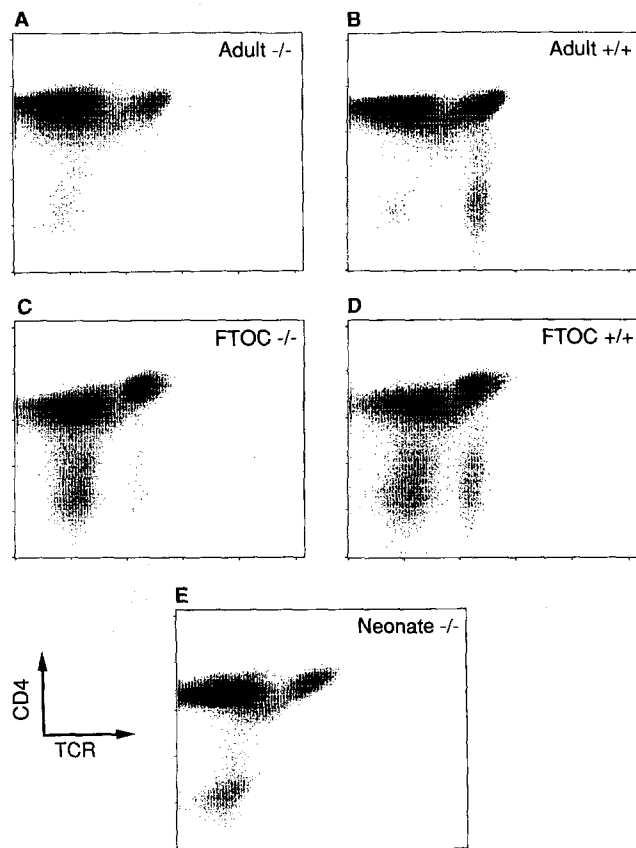
## Results and Discussion

### T Cells Recognize Peptide Antigen on $\beta_2$ M-deficient Cells.

In the class I antigen presentation pathway, a trimolecular complex of class I H chain,  $\beta_2$ M, and peptide are formed in the endoplasmic reticulum and efficiently transported to the cell surface. In cells lacking  $\beta_2$ M this complex is not formed and H chain expression on the cell surface is greatly impaired (2, 10, 11). Tumor cells deficient in  $\beta_2$ M cannot present endogenously produced viral antigens but they are able to present peptide antigens when the peptide is supplied exogenously with  $\beta_2$ M (7). To directly compare the ability of  $\beta_2$ M-deficient and wild type cells to present peptide antigens, we titrated the natural peptide epitope for an OVA-specific CTL. Fig. 1 shows CTL recognition of H-2<sup>b</sup> targets from either normal mice or mice deficient in  $\beta_2$ M. Both cells can be targets for CTL after pulsing with a K<sup>b</sup> binding



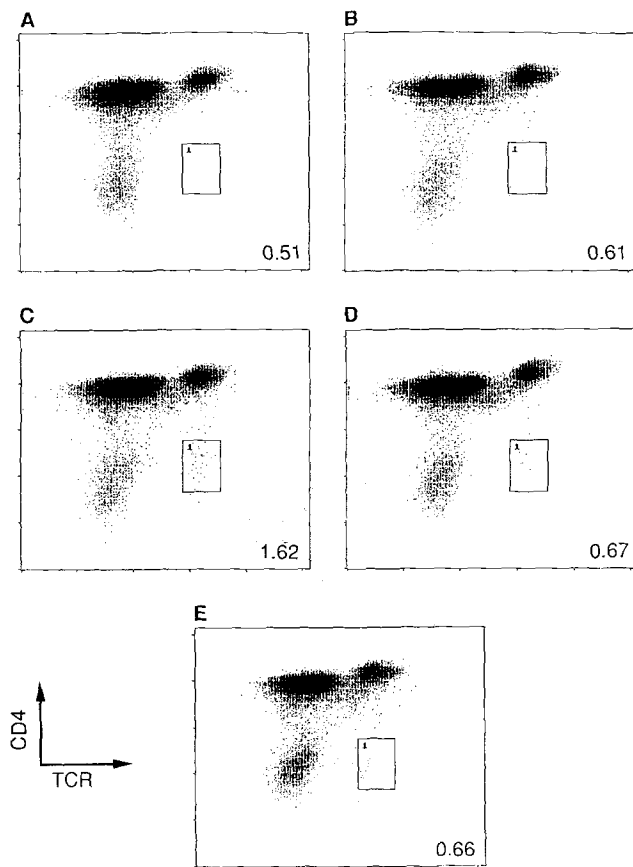
**Figure 1.** Sensitivity of  $\beta_2$ M deficient cells to CTL in the presence of peptides and  $\beta_2$ M. Lymphoblasts from C57BL/6 or  $\beta_2$ M<sup>-/-</sup> mice were incubated with various concentrations of OVA 257-264 peptide, in the presence or absence of FCS as a source of exogenous  $\beta_2$ M. Cells were incubated for 1 h at 37°C in the presence of <sup>51</sup>Cr. After washing, GA4 CTL were added. Specific lysis was determined after 4 h. Serum-free medium was used at every step except where indicated.



**Figure 2.** Adult, neonatal, and FTOC thymocytes from  $\beta_2$ M<sup>-/-</sup> animals are deficient in mature CD8<sup>+</sup> T cells. (A and B) Adult thymocytes were stained for CD4 and TCR- $\alpha/\beta$ . TCR- $\alpha/\beta$  staining (x-axis) and CD4 staining (y-axis) are presented on a logarithmic scale. (C and D) Thymocytes recovered from FTOC. (E) Neonatal (day 3) thymocytes from  $\beta_2$ M<sup>-/-</sup> mice. Each panel is from an individual animal, and is representative of at least three animals in each group.

peptide antigen and FCS as a source of  $\beta_2M$ . Normal cells do not require the addition of FCS, whereas the  $\beta_2M$ -deficient cells do. Under these conditions, the concentration of peptide required for 50% lysis of  $\beta_2M$ -deficient cells is about 100-fold more than for wild type cells. Purified human  $\beta_2M$  also provided targeting (data not shown).

**CD8 Development Is Altered in FTOC of  $\beta_2M$ -deficient Mice.** The above data shows that  $\beta_2M$ -deficient cells can present exogenous peptide antigen to T cells when provided with  $\beta_2M$ . We reasoned that CD8 development in FTOC from these mice might then be susceptible to alteration by addition of exogenous peptide in the presence of  $\beta_2M$ . First it was necessary to determine whether FTOC from  $\beta_2M^{-/-}$  mice showed a block in CD8<sup>+</sup> development. Fig. 2, A and B shows FACS<sup>®</sup> profiles of TCR and CD4 expression in adult thymuses of normal and  $\beta_2M^{-/-}$  mice. As was previously shown, these mice are profoundly deficient



**Figure 3.** Effect of class I MHC binding peptides on  $\beta_2M^{-/-}$  FTOC.  $\beta_2M^{-/-}$  lobes were cultured for 10 d in the following: (A) serum free medium (SFM) alone; (B) SFM plus 10% FCS; (C) SFM plus 10% FCS plus 100  $\mu\text{g/ml}$  each OVA 257-264 ( $K^b$  binding) and Flu nucleoprotein 366-374 ( $D^b$  binding) peptides; (D) SFM plus the above peptides, but with no FCS; and (E) SFM plus 10% FCS plus 200  $\mu\text{g/ml}$  listeriolysin-O 211-219 peptide (no  $K^b$  or  $D^b$  binding). Thymocytes recovered from FTOC lobes were stained as in Fig. 2. The number in the lower right corner is the percentage of CD4<sup>+</sup> TCR<sup>hi</sup> cells in the boxed area. 100,000 cells were analyzed per sample. Each dot represents three cells.

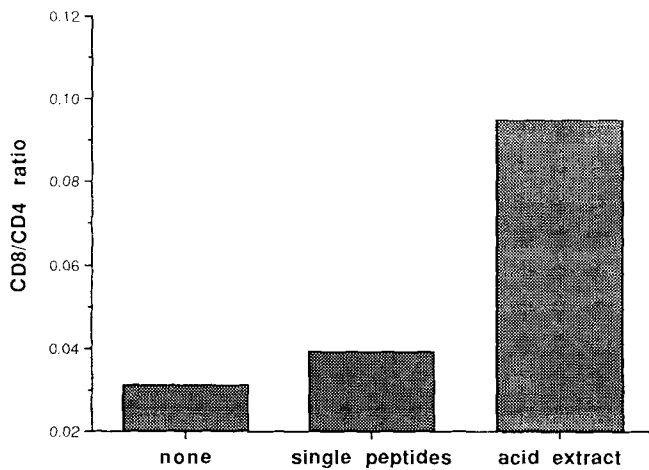
in CD8<sup>+</sup> CD4<sup>-</sup> TCR<sup>hi</sup> mature T cells. Likewise, thymic lobes from these strains (Fig. 2, C and D) develop similar profiles after 10 d in culture. This indicates that the CD8<sup>+</sup> cells of mature phenotype which develop in this culture system, are in fact the result of positive selection on class I molecules. Other investigators have suggested that cells with a mature phenotype can develop in the embryonic thymus in the absence of a positive selecting signal (12, 13). Our data argue that this is not the case.

Though the mutant mice show a deficiency of mature CD8<sup>+</sup> T cells in FTOC (Fig. 2 C), it is not as severe as in adult mice (Fig. 2 A). For example, the CD8<sup>+</sup> to CD4<sup>+</sup> ratio amongst TCR<sup>hi</sup> cells in adult  $\beta_2M^{-/-}$  mice is 0.01, whereas in FTOC it is 0.03. This threefold difference is small but highly reproducible, and is not due to comparing adult to fetal thymuses, since 3-d-old (roughly age matched to FTOC) mice have a mature CD8/4 ratio of 0.015 (Fig. 2 E). What factors allow for this slightly greater CD8<sup>+</sup> development in vitro as compared to in vivo is not known.

**Class I Binding Peptides Affect CD8<sup>+</sup> Development only in the Presence of  $\beta_2M$ .** Next we asked whether class I binding peptides added to these cultures could effect CD8<sup>+</sup> development. For all of these experiments the basal medium was serum free. Thymic lobes from normal mice developed identically in this medium compared to medium containing 10% FCS (data not shown). Fig. 3 shows that with lobes from  $\beta_2M^{-/-}$  mice, no difference was seen when 10% FCS was included in the medium (compare Fig. 3, A and B).

When class I binding peptides (in this experiment 100  $\mu\text{g/ml}$  each of a  $K^b$  binding octamer and a  $D^b$  binding nonamer) were included, a small increase was seen in the mature CD8<sup>+</sup> subset (Fig. 3 C). There was no change seen in the other subsets. In comparing between experiments, the overall extent of T cell development can vary. To control for this we have compared the CD8 to CD4 ratio amongst TCR<sup>hi</sup> cells. In four experiments, the mean ratio was  $.030 \pm .002$  when cultured with FCS alone,  $.046 \pm .012$  when cultured with peptide and FCS. This increase was dependent on the addition of  $\beta_2M$  (Fig. 3 D) and an equivalent concentration of a peptide which does not bind H-2b class I molecules had no effect, even in the presence of  $\beta_2M$  (Fig. 3 E). These data demonstrate that positive selection is dependent on class I binding peptides.

**Complex Mixtures of Peptides Are More Efficient.** Although single peptides are sufficient to sensitize  $\beta_2M$ -deficient targets for CTL, and to cause some CD8<sup>+</sup> development in FTOC, they may not provide high enough levels of conformed class I complexes to mediate this selective event efficiently. Alternatively, it is possible that single peptides could never select the entire TCR repertoire, and that a complex mixture of peptides is required. To test this hypothesis, we added a mixture of peptides to FTOC. As a source of heterogenous class I binding peptides we used the low molecular weight material from an acid extract of spleen cells. Fig. 4 shows that this material is considerably more effective in inducing CD8<sup>+</sup> development than are single peptides. The data is expressed as the ratio of CD4 single positive (SP) to CD8 SP



**Figure 4.** Effect of peptide extracts on  $\beta_2M^{-/-}$  FTOC. Lobes from  $\beta_2M^{-/-}$  mice were cultured for 10 d in medium with 10% FCS and a mixture of OVA 257-264 and Flu nucleoprotein 366-374 or an acid extract from C57BL/6 spleen, each at a final concentration of 200  $\mu\text{g}/\text{ml}$ . Thymocytes recovered from the lobes were stained as in Fig. 2. Data are expressed as the ratio of CD8<sup>+</sup> to CD4<sup>+</sup> cells amongst the TCR<sup>hi</sup> cells of three to four pooled lobes in each group. The experiment was repeated four times with similar results.

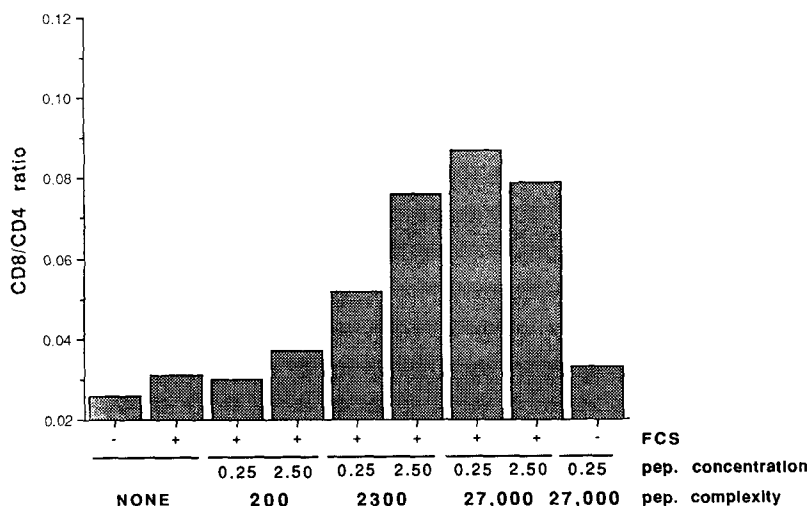
amongst TCR<sup>hi</sup> cells. If we consider the total yield of cells per lobe in this particular experiment, the yield of CD4 SP mature cells per lobe was  $8.9 \times 10^4$  in the control and  $8.5 \times 10^4$  in the presence of the acid extract, whereas the yield of CD8 SP mature cells went from  $2.6 \times 10^3$  to  $8.7 \times 10^3$ . Though the total protein concentration in the acid extract is equivalent to the amount of single peptides used, the concentration of class I binding peptides in the acid extract is presumably a small percentage of that. Despite the overall lower amount of class I binding peptides in the acid extract as compared to single peptides, there is a greater effect on CD8<sup>+</sup> selection. This effect was reduced by 70% when no  $\beta_2M$  was added.

The extract in Fig. 4 is from C57BL/6 spleen. However, extracts from BALB/c spleen are equally effective at medi-

ating CD8<sup>+</sup> development (data not shown). This may be due to the contribution of non-MHC bound peptides that are generated during the acid extraction protocol or to peptides that are shared by MHC alleles.

To further investigate whether peptide complexity was a critical component of CD8<sup>+</sup> development, we used a recombinant system for generating class I binding peptides. Heterogenous octamer peptides were produced in *E. coli* as a fusion protein with maltose binding protein. The degenerate oligonucleotide used to generate this library encoded peptides of the following composition: SXIXFXXL, where X is any of 20 amino acids. These peptides have the K<sup>b</sup> anchor residues conserved while varying the residues predicted to contact the TCR. By using various numbers of colonies to begin the preparation, we controlled the heterogeneity of the peptide mixture produced. Mixtures consisting of an estimated 200, 2,300, or 27,000 K<sup>b</sup> binding peptides were produced and purified on reverse phase HPLC. To assess how well these peptide mixtures bound K<sup>b</sup> we used the RMA-S stabilization assay (14) and found that the resultant mixtures were as effective as single H-2K<sup>b</sup> octamer peptides at binding to K<sup>b</sup> (data not shown). These mixtures were then tested for their ability to mediate CD8<sup>+</sup> development in  $\beta_2M^{-/-}$  FTOC. Fig. 5 shows that mixtures of greater complexity are more efficient at mediating CD8 development than those of lower complexity. The highest concentration of peptides used in this experiment was 2.50  $\mu\text{g}/\text{ml}$ , almost 100-fold lower than that used with single peptides. At this concentration, no development was seen with the single peptides used in Fig. 3 (data not shown). The enhancement of CD8<sup>+</sup> maturation was entirely dependent on exogenous  $\beta_2M$  (Fig. 5).

These data show that the induction of CD8<sup>+</sup> T cell development is more efficient on a mixture of class I-peptide complexes and suggest that TCRs require specific self-peptide/MHC ligands to be positively selected. This has been suggested in previous experiments in which it has been shown that in TCR transgenic mice (15) or in normal mice (16) positive selection of a specific TCR or a specific response to foreign antigen can be disrupted by using K<sup>b</sup> molecules with



**Figure 5.** Effect of recombinant peptide mixtures of varying complexity on  $\beta_2M^{-/-}$  FTOC. FTOC was performed as described above. Octamer mixes of various complexities were purified by HPLC and added at a final concentration of 2.50 or 0.25  $\mu\text{g}/\text{ml}$  with or without FCS. Data are expressed as the ratio of CD8<sup>+</sup> to CD4<sup>+</sup> cells amongst the TCR<sup>hi</sup> cells of two to four pooled lobes. A repeat experiment gave similar results.

changes that are thought to affect only the peptide binding groove. Of ultimate interest is the nature of a positively selecting ligand for a given TCR and how that MHC-self-peptide complex is related to the MHC-foreign peptide com-

plex. This model system will be useful in identifying peptides that can mediate positive but not negative selection in TCR transgenic mice.

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