Mature T Cell Reactivity Altered by Peptide Agonist that Induces Positive Selection

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Summary

Recent studies have investigated how defined peptides influence T cell development. Using a T cell receptor–transgenic β_2-microglobulin–deficient model, we have examined T cell maturation in fetal thymic organ cultures in the presence of various peptides containing single-alanine substitutions of the strong peptide agonist, p33. Cocultivation with the peptide A4Y, which contains an altered T cell contact residue, resulted in efficient positive selection. Several in vitro assays demonstrated that A4Y was a moderate agonist relative to p33. Although A4Y promoted positive selection over a wide concentration range, high doses of this peptide could not induce clonal deletion. Thymocytes maturing in the presence of A4Y were no longer able to respond to A4Y, but could proliferate against p33. These studies demonstrate that (*a*) peptides that induce efficient positive selection at high concentrations are not exclusively antagonists; (*b*) some agonists do not promote clonal deletion; (*c*) positive selection requires a unique T cell receptor–peptide–major histocompatibility complex interaction; and (*d*) interactions with selecting peptides during T cell ontogeny may define the functional reactivity of mature T cells.

TCRs expressed on maturing thymocytes interact with peptide–MHC complexes on thymic stromal cells and transmit signals that lead to either positive or negative T cell selection (1–3). Positive selection is an active process that rescues self-MHC–restricted thymocytes from programmed cell death. In contrast, negative selection tolerizes potentially autoreactive T cells, either through clonal deletion or unresponsiveness. Clonal deletion physically removes thymocytes by inducing apoptosis, as compared with unresponsiveness, which modifies developing T cells so that they can no longer respond against the tolerizing antigen. Since positive and negative selection shape the TCR repertoire and define the basis of self-/non–self-antigens, much research has focused on understanding T cell development. Studies have addressed how clonotypic TCRs expressed on $CD4^+CD8^+$ double-positive thymocytes can distinguish between these two selection events. Although it has been demonstrated that peptides are involved in positive and negative selection (4, 5), it remains controversial whether the selecting ligand has a qualitative or quantitative role in determining the fate of the developing T cell.

Recently, altered peptide ligands have been identified that can inhibit some or all mature T cell effector functions (6–10). Antagonist peptides are defined as ligands that engage TCRs and actively inhibit biological responses. Partial agonists are closely related peptides that stimulate a subset of T cell effector functions (11). This is in contrast with agonist peptides, which induce a complete range of T cell responses. Studies by Hogquist et al. (12, 13) and Jameson et al. (14) showed a correlation between peptide antagonists and positive selection. These results suggest that a positively selecting peptide is qualitatively different from a peptide agonist and that the selecting ligand shares features characteristic of partial agonists and antagonists. However, other studies have demonstrated that low concentrations of a strong peptide agonist can promote positive selection, whereas higher concentrations of the same peptide lead to negative selection (15, 16), suggesting that quantitative differences in thymocyte–stromal cell interactions determine the fate of the maturing T cell.

The nature of the selecting ligand has direct implications on the type of signals generated during thymocyte selection and has fostered several models of T cell development (17–22). Recent experiments have demonstrated that altered peptide ligands generate a different ratio of intracellular signals compared with agonist-induced T cell activation (23, 24). These biochemical alterations support the hypothesis that a positively selecting antagonist peptide transmits a

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/96/03/1093/12 $2.00
Volume 183 March 1996 1093–1104

unique signal to an immature T cell, allowing for further maturation. For instance, a selecting ligand may induce a specific TCR conformation that conveys a partial signal, resulting in positive selection (3). Models supporting the correlation between incomplete signals generated by antagonists or partial agonists and positive selection are based on the inability of these peptides to activate mature T cells. It is equally feasible that selecting peptides simply contribute to the affinity of TCR–MHC interactions and do not possess novel signaling properties. In an attempt to address these various issues, we have examined how minor changes of a natural peptide ligand alter TCR-transgenic thymocyte selection.

T cell development was examined using a TCR-transgenic mouse model (327 line) specific for lymphocytic choriomeningitis virus glycoprotein peptide (LCMV-gp)[1] presented by the MHC class I molecule, H-2D^b. TCR-transgenic β_2-microglobulin (β_2m) deficient (TCR $\beta_2m^{-/-}$) mice were generated, and fetal thymic lobes from these mice were cultured in the presence of exogenous β_2m and various peptides containing single alanine substitutions of the strong peptide agonist p33. Our results describe a novel peptide agonist capable of inducing positive selection but not clonal deletion. This selection process seems to involve a unique TCR–peptide–MHC engagement that is not restricted to agonist or antagonist peptides. In addition, our data demonstrate that the interactions between TCR and peptide–MHC during development modify the reactivity and the ability of the mature T cell to respond to foreign peptide antigens.

Materials and Methods

Mice. TCR-transgenic mice were previously generated using α and β chains isolated from CTL clone P14, which recognized the LCMV glycoprotein (peptide p33-41) presented by H-2D^b (25). This line was crossed with H-2^b $\beta_2m^{-/-}$ mice (26). TCR-transgenic F_1 mice were subsequently backcrossed with $\beta_2m^{-/-}$ mice to obtain TCR $\beta_2m^{-/-}$ (H-2^b) mice. Progeny were typed for transgenic TCR and homozygous β_2m disruption by staining peripheral blood with mAbs at 4°C in PBS containing 2% FCS, 0.2% NaN_3, and 20 mM EDTA. TCR-transgenic cells were detected with rat anti–mouse Vβ8.1 (KJ16) mAb (27) followed by FITC-conjugated goat anti–rat mAb (Tago Inc., Burlingame, CA). $\beta_2m^{-/-}$ mice were screened for a lack of CD8$^+$ cells using rat anti–mouse CD8 (YTS 169.4) mAb (28) followed by FITC-conjugated goat anti–rat mAb. After the second Ab incubation period, RBC were lysed using 1× FACS® lysing solution (Becton Dickinson & Co., Mountain View, CA). TCR$^+$ RAG2$^{-/-}$ mice were previously generated (29).

Fetal Thymic Organ Cultures (FTOC). Timed breedings were established between TCR $\beta_2m^{-/-}$ H-2^b males and $\beta_2m^{-/-}$ H-2^b females. At day 16 of gestation, females were killed, and thymic lobes were removed from the fetuses. DNA was extracted from embryonic tails so that transgenic fetuses could be determined using primers (Vα2 - CTG ACC TGC AGT TAT GAG GAC AGC AC and Cα - CGA GGA TCC TTT AAC TGG TAC ACA GCA GG) specific for the Vα2 TCR transgene. The fetal thymic lobes were placed on 0.8-μm polycarbonate filters (Costar Corp., Cambridge, MA), which floated on 1 ml of IMDM, 1× Nutridoma-SP (Boehringer Mannheim Corp., Indianapolis, IN), 5×10^{-5} M 2-ME, penicillin, streptomycin, 2 mM glutamine, 2.5 μg human β_2m (Sigma Chemical Co., St. Louis, MO), and designated peptides. These lobes were then cultured for 6 d at 37°C, during which time the medium was changed daily. After this incubation period, the thymic lobes were teased apart and stained with mAbs at 4°C in PBS containing 2% FCS and 0.2% NaN_3. Three-color analysis was done with rat anti–mouse PE-conjugated anti-CD4 (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada), FITC-conjugated anti-CD8 (Cedarlane Laboratories Ltd.), and biotinylated anti-Vα2 (B20.1) (PharMingen, San Diego, CA), or biotinylated anti–heat stable antigen (HSA) (M1/69) (PharMingen). Biotinylated antibodies were detected with streptavidin–red 670 (GIBCO BRL, Gaithersburg, MD).

Flow Cytometry. All flow cytometric analysis was performed on a FACScan® instrument (Becton Dickinson & Co.). Samples were gated for live cells based on forward and side scatter parameters (10,000 events/sample) and analyzed using Lysys II software (Becton Dickinson & Co.).

Peptides. The peptides p33 (KAVYNFATM), A4Y (KAVANFATM), A1K (AAVYNFATM), A3V (KAAYNFATM), A6F (KAVYNAATM), A8T (KAVYNFAAM), S4Y (KAVSNFATM), adenovirus peptide AV (SGPSNTPPEI), and LCMV nucleoprotein 118-127 (RPQASGVYMG) were synthesized by a solid-phase method using the Fmoc/tBu-based protocol. Chain assembly was carried out at the Amgen Institute using a single-coupling program on an ABI-431 instrument. The crude products were purified on a reverse-phase preparative HPLC column (C4; Vydac, Hesperia, CA). Homogeneity of the final products was assessed by analytical HPLC. Characterization was provided by amino acid analysis and electrospray mass spectometry.

Peptide Modeling. H-2D^b models of p33 and A4Y were modeled using both published (30) and unpublished (Thomson, C.T., S.G. Nathenson, and J.C. Sacchettini, unpublished data) H-2D^b crystal structure coordinates with initial results obtained using the homology module of Insight II software programs from Biosym Technologies (San Diego, CA). Models were refined for 1,000 cycles of steepest descents energy minimization.

Peptide-binding Assay. In peptide-pulsing experiments, 10^6 RMA-S cells, which were previously cultured overnight at 29°C in RPMI plus 10% FCS, were incubated with various concentrations of peptide at 29°C for 30 min. These cells were then transferred to a 37°C incubator for 3 h, after which the cells were washed and stained with anti–H-2D^b mAb from tissue culture supernatant (B22.249) (31, 32) and then FITC-conjugated rat anti–mouse Ig (Sigma Chemical Co.). RMA-S cells were incubated with LCMV nucleoprotein 118-127 (H-2^d restricted) (33) to determine background H-2D^b expression.

Cytolytic Assay. MC57G fibroblast target cells (H-2^b) were prepulsed with peptides (10^{-6} M) for 3 h at 37°C and pulsed with ^{51}Cr (Dupont NEN, Boston, MA) for 1 h at 37°C. These cells (10^4/well) were then incubated for 5 h at 37°C in 96-well round-bottom plates with serial dilutions of spleen cells from TCR-transgenic mice that had been primed intravenously 4 d earlier with 5×10^6 PFU LCMV-Armstrong. The supernatant was subsequently counted using a gamma counter (model 1282 Compugamma CS Universal; LKB Wallac, Turku, Finland). The percentage of specific lysis was calculated as (experimental release − spontaneous release)/(total release − spontaneous release) × 100.

[1] *Abbreviatons used in this paper:* Av, adenovirus; β_2m, β_2-microglobulin; FTOC, fetal thymic organ culture; HSA, heat stable antigen; LCMV-gp, lymphocytic choriomeningitis virus glycoprotein peptide.

Spontaneous release from peptide-coated MC57G target cells was <20% in all experiments.

Primary In Vitro Generation of Cytotoxicity. TCR-transgenic spleen cells (3×10^6/well) were cocultured with irradiated C57Bl/6J macrophages coated with p33, A4Y, or AV (10^{-6}, 10^{-8} or 10^{-10} M) at 37°C in IMDM, 10% FCS, 5×10^{-5} 2-ME, penicillin, and streptomycin. 3 d later these cultures were harvested and incubated with EL-4 target cells (H-2^b) that had been prepulsed with peptides (10^{-6} or 10^{-8} M) for 3 h and pulsed with ^{51}Cr (Dupont NEN) for 1 h at 37°C. Peptide-specific lysis was determined using a standard 5-h chromium release assay. Spontaneous release from peptide-coated EL-4 target cells was <20% in all cases.

Proliferation Assays. Spleen cells (10^5/well) from TCR-transgenic or TCR-transgenic RAG2$^{-/-}$ mice were incubated in triplicate in 96-well flat-bottom plates with 2×10^4/well irradiated C57Bl/6J (H-2^b) macrophages that had been prepulsed with various concentrations of peptide for 3 h at 37°C. After 48 h of cocultivation, the cells were pulsed with 1 μCi of [^{3}H]-thymidine (Amersham Corp., Arlington Heights, IL) for 16 h. Cells were harvested and counted on a direct beta counter (Matrix96; Canberra Packard Canada Ltd., Mississauga, Ontario, Canada).

GM-CSF/IL-3 Assay. Supernatant from TCR-transgenic spleen cells (10^5/well) cocultured with irradiated, peptide-coated C57Bl/6J (H-2^b) macrophages was collected and transferred to 96-well flat-bottom microtiter plates containing washed FDC-P1 cells (10^4/well) that had not received fresh medium containing IL-3 for 3–4 d. The cells were incubated for 24 h and then pulsed with 1 μCi of [^{3}H]-thymidine for 16 h at 37°C. Cells were harvested and counted on a Matrix96 direct beta counter. A standard curve was generated using serial dilutions of recombinant mouse IL-3 (Genzyme Corp., Cambridge, MA).

TCR Antagonist Assay. Irradiated C57Bl/6J macrophages (2×10^4/well) were prepulsed with 10^{-8} M p33 for 3 h at 37°C, after which the cells were washed three times and pulsed with serial dilutions of A4Y or S4Y (KAVSNFATM). These cells were incubated for another 3 h at 37°C, washed, and then cultured with TCR-transgenic spleen cells (10^5/well) at 37°C for 48 h. These cultures were then pulsed with 1 μCi of [^{3}H]-thymidine for 16 h, after which time the cells were harvested and counted on a Matrix96 direct beta counter.

FTOC Proliferation Assay. Cultured thymic lobes were teased apart and stained at 4°C in PBS containing 2% FCS with FITC-conjugated anti-CD8 (Cedarlane Laboratories Ltd.) and PE-conjugated anti-CD4 (Cedarlane Laboratories Ltd.). These cells were then sorted using a FACStar® Plus instrument (Becton Dickinson & Co.) to collect CD8$^+$ thymocytes. Irradiated spleen cells from a C57Bl/6J mouse were prepulsed with 10^{-8} M AV, A4Y, or p33 for 3 h at 37°C, washed, and distributed in triplicate on a flat-bottom 96-well plate at a concentration of 10^5 cells/well. CD8$^+$ thymocytes (3×10^4/well) resuspended in IMDM, 10% FCS, penicillin, streptomycin, and 5×10^{-5} M 2-ME were then added to these wells. The cells were cultured at 37°C for 4 d, pulsed with 1 μCi of [^{3}H]-thymidine for 16 h, and harvested as described.

Results

A Modified Peptide Containing a Single Alanine Substitution Mediates Efficient Positive Selection. To examine how peptides influence thymocyte selection, fetal thymic lobes from TCR β_2m$^{-/-}$ mice were cultured in the presence of β_2m and various peptides containing a single alanine substitution of the strong peptide agonist, p33 (KAVYNFATM). One of the peptide variants, A4Y (KAVANFATM), was notable for its ability to efficiently induce TCR-transgenic thymocyte maturation. This was characterized by a skewed CD8$^+$ population that expressed high levels of the transgenic TCR as demonstrated by three color flow cytometry (Fig. 1 *a*). When TCR β_2m$^{-/-}$ fetal thymic lobes were incubated in vitro with exogenous β_2m, few CD8$^+$ cells were detected, and these cells did not express high levels of the transgenic receptor. A control adenovirus (AV) peptide (SGPSNTPPEI), which is known to efficiently bind to H-2D^b (34, 35) also did not induce maturation of the LCMV-specific TCR^{hi} thymocytes. Three-color analysis of positively selected TCR^{hi} thymocytes demonstrated that CD8$^+$ thymocytes expressed low levels of HSA, characteristic of mature thymocytes (36) (Fig. 1 *b*). As shown in Table 1, A4Y generated four to five times as many CD8$^+$ thymocytes compared with negative control FTOCs. In addition, the majority of CD8$^+$ thymocytes collected from AV-treated organ cultures stained HSA^{hi}, indicating that these cells had not yet undergone positive selection (data not shown). These experiments demonstrate that the peptide A4Y positively selected the transgenic TCR with an efficiency comparable to wild-type TCR β_2m$^+$ lobes cultured in media alone. Therefore, positive selection of the transgenic TCR is efficiently mediated by the altered peptide antigen A4Y.

Alterations at Position 4 Potentially Affects a TCR Contact Residue. To characterize this peptide, molecular models of A4Y and p33 bound in the groove of H-2D^b (30) were generated and compared. Based on the peptide sequences, it was predicted that the tyrosine amino acid side chain at position 4 was directed away from the MHC class I molecule and interacted with the TCR (Fig. 2 *a*). This is consistent with evidence from in vivo studies of variant LCMV

Table 1. *Total and CD8$^+$ Thymocyte Numbers from TCR $\beta_2m^{-/-}$ and TCR $\beta_2m^{+/+}$ FTOCs Treated with Various Peptides*

Peptide	Total cell number*	CD8$^+$ cell number*
TCR β_2m$^{-/-}$ (n = 4)		
No peptide	$7.5 \pm 0.8 \times 10^5$	$6.0 \pm 0.6 \times 10^4$‡
AV (10^{-6} M)	$5.8 \pm 0.7 \times 10^5$	$5.2 \pm 0.6 \times 10^4$‡
A4Y (10^{-6} M)	$7.8 \pm 0.3 \times 10^5$	$2.3 \pm 0.1 \times 10^5$
TCR β_2m$^{-/-}$ (n = 5)		
A4Y (10^{-4} M)	$7.2 \pm 1.4 \times 10^5$	$2.2 \pm 0.4 \times 10^5$
A4Y (10^{-6} M)	$6.3 \pm 1.2 \times 10^5$	$2.4 \pm 0.4 \times 10^5$
A4Y (10^{-9} M)	$6.1 \pm 0.8 \times 10^5$	$1.7 \pm 0.2 \times 10^5$
TCR β_2m$^{+/+}$ (n = 8)		
No peptide	$1.2 \pm 0.3 \times 10^6$	$3.2 \pm 0.8 \times 10^5$
A4Y (10^{-6} M)	$9.0 \pm 3.0 \times 10^5$	$2.2 \pm 0.7 \times 10^5$
p33 (10^{-6} M)	$1.0 \pm 0.2 \times 10^5$	$1.0 \pm 0.2 \times 10^4$

*Data ± SD.
‡Predominantly immature CD8$^+$ HSA^{hi} thymocytes.

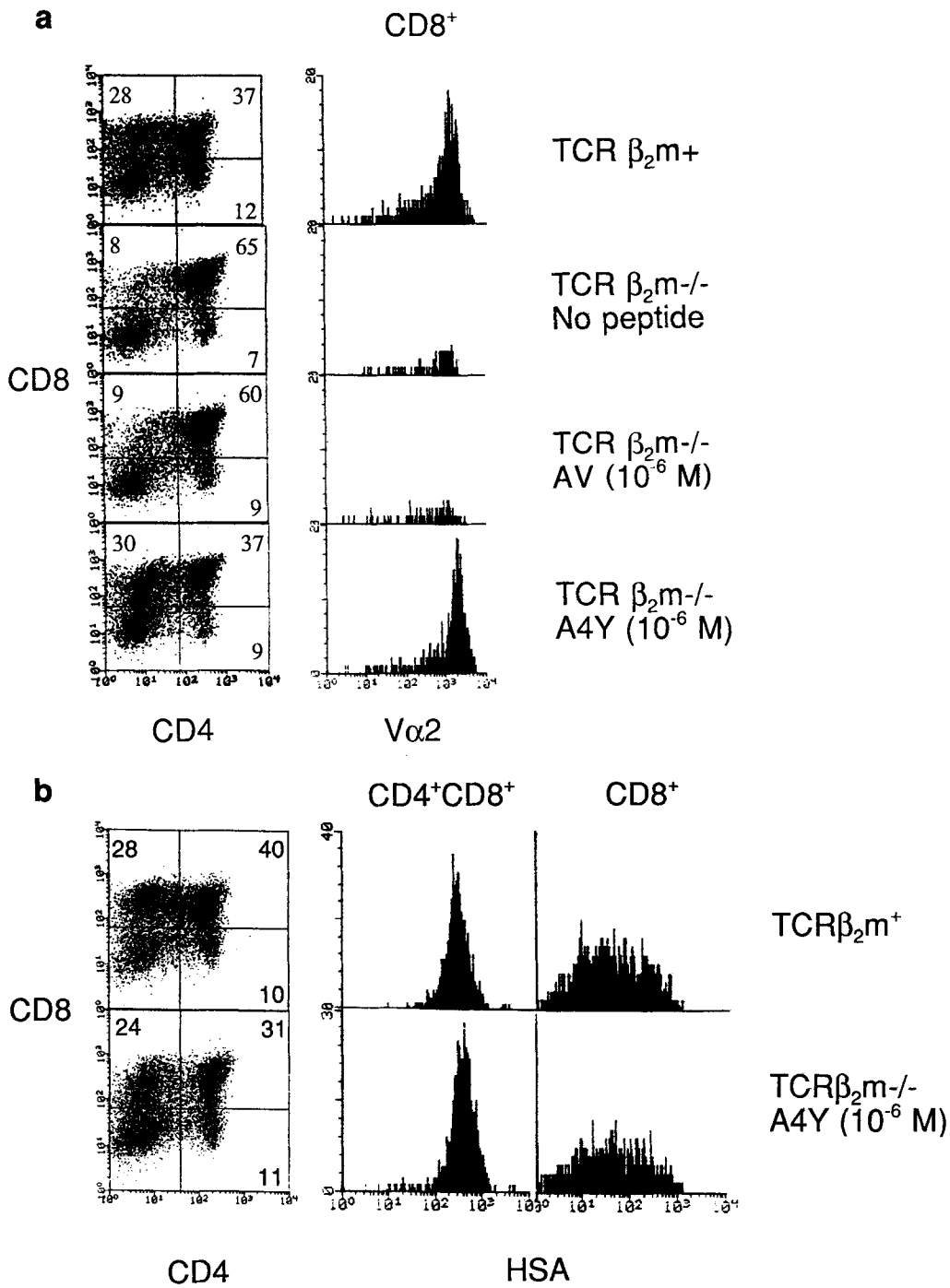
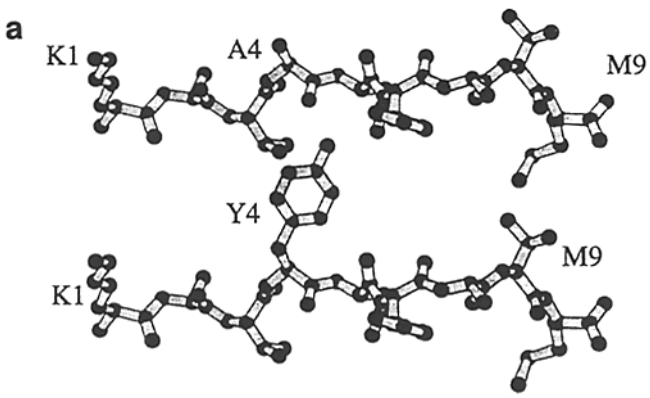


Figure 1. Addition of A4Y results in efficient positive selection of transgenic thymocytes in TCR $\beta_2m^{-/-}$ FTOC. (*a*) Three-color analysis of TCR $\beta_2m^{-/-}$ thymic lobes cultured without peptide, AV (a control H-2D^b–restricted adenovirus peptide) or A4Y were stained with antibodies specific for CD4, CD8, and Vα2. CD8$^+$ cells were gated, and the profiles of Vα2 expression are shown. This experiment was repeated 10 times, producing similar results. (*b*) TCR $\beta_2m^{+/+}$ and TCR $\beta_2m^{-/-}$ fetal thymic lobes cultured with A4Y were stained with antibodies specific for CD4, CD8, and HSA. HSA profiles are shown from gated CD4$^+$CD8$^+$ or CD8$^+$ cells. These data are representative of four separate experiments.

strains isolated from TCR-transgenic mice. These virus variants escape the immune surveillance of the transgenic TCR by altering the p33-41 epitope at positions 3 and 4 (37). Therefore, the alanine substitution at this position is predicted to affect TCR–peptide interactions.

Previous experiments have shown that asparagine and methionine are anchor residues that bind to H-2D^b (38). Although these amino acids were not modified in A4Y, peptide binding to H-2D^b was tested using the murine lymphoma cell line RMA-S (39). RMA-S cells incubated with various concentrations of p33, A4Y, or control AV showed similar increases in H-2D^b staining (Fig. 2 *b*), dem-



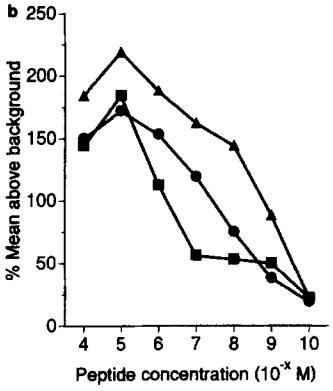


Figure 2. Peptide A4Y contains an alanine substitution that is likely to affect a TCR contact residue. (*a*) The peptides A4Y and p33 are shown as modeled in the binding groove of an H-2D^b molecule (side view). The TCR contact residues are oriented toward the top of the page. (Figure produced by MOLSCRIPT). (*b*) The ability of peptides to rescue H-2D^b surface expression on RMA-S cells was characterized by percentage increase in mean fluorescence relative to the control peptide, LCMV nucleoprotein 118-127 (H-2^d restricted) (33). RMA-S cells were treated with different concentrations of p33 (■), A4Y (●), or AV (▲). Similar results were obtained when this experiment was repeated four times.

onstrating that these peptides have a comparable ability to bind to the MHC class I molecule.

The Peptide A4Y Displays Agonistic Properties. The ability of the transgenic TCR to interact with A4Y and p33 was compared in several in vitro assays. To determine whether the transgenic TCR could recognize A4Y, TCR β_2m^+ mice were immunized with LCMV, and activated spleen cells were incubated with target cells pulsed with p33, A4Y, or an irrelevant adenovirus peptide AV (Fig. 3). Comparable specific lysis was seen in the presence of targets coated with p33 or A4Y, showing that the activated transgenic T cells could easily recognize both peptides.

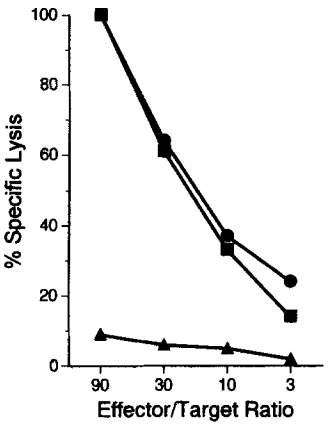


Figure 3. Activated TCR-transgenic T cells recognize the peptides p33 and A4Y. The cytotoxic activity of LCMV-primed transgenic T cells was tested against target cells coated with p33 (■), A4Y (●), or AV (▲). These data are representative of CTL analysis from four mice.

This cytotoxic assay addresses the ability of activated T cells to recognize the altered peptides, but does not examine whether these peptides can directly activate the transgenic T cell population. For this reason, TCR-transgenic spleen cells from naive mice were cocultured with macrophages coated with different concentrations (10^{-6}, 10^{-8} or 10^{-10} M) of the peptides p33, A4Y, and AV. After 3 d, serial dilutions of each well were incubated with target cells coated with 10^{-6} or 10^{-8} M p33, A4Y, or AV (Fig. 4). Comparable cytolytic activity specific for p33 or A4Y was detected using transgenic T cells previously cultured with p33. The percentage of cytotoxicity was slightly reduced when activated in the presence of macrophages pulsed with low concentrations of p33 peptide (10^{-10} M). However, transgenic T cells cocultivated in the presence of various concentrations of A4Y were only able to weakly lyse targets expressing either p33 or A4Y. No specific lysis was detected using control cultures incubated with irrelevant peptide. Visual examination of cells cultured under these various conditions suggested that strong proliferation only occurred in the wells containing the peptide p33. These experiments show that both p33 and A4Y can initiate cytotoxic effector functions. However, the relatively limited cytotoxic response induced by A4Y-primed T cells suggests that this peptide did not interact with the transgenic TCR as efficiently as the wild-type peptide.

It is possible that A4Y could not induce optimal cytotoxicity because of a reduced ability to trigger the proliferation of naive transgenic T cells. Therefore, proliferation assays were done by cocultivating transgenic spleen cells with macrophages pulsed with various concentrations of different peptides (Fig. 5 *a*). A significantly stronger proliferative response was detected from T cells cultured in the presence of p33 compared with A4Y. Interestingly, A4Y induced similar levels of T cell activation relative to other alanine-substituted variants of p33 that were less efficient at promoting transgenic thymocyte maturation.

To confirm that these in vitro observations were specific for the transgenic TCR and not due to the expression of endogenous TCR-α chains, we performed a proliferation assay using splenocytes from TCR RAG2$^{-/-}$ mice. Since these T cells do not express endogenous TCR-α chains, Fig. 5 *b* verifies that the observed results were in fact due to the transgenic TCR. These experiments show that A4Y can elicit a TCR-transgenic proliferative response, albeit not to the same extent as p33.

Cytokine assays were performed to further investigate A4Y's ability to stimulate TCR-transgenic T cell effector functions. As shown in Fig. 6, A4Y could induce GM-CSF/IL-3 production. Cytokine levels were reduced when compared with supernatant from p33-stimulated cultures; however, this reduction paralleled the previously observed decrease in proliferation. T cells cultured with a control peptide did not produce detectable levels of IL-3. Similar results were found when IL-2 production was analyzed (data not shown). This work demonstrates that A4Y in-

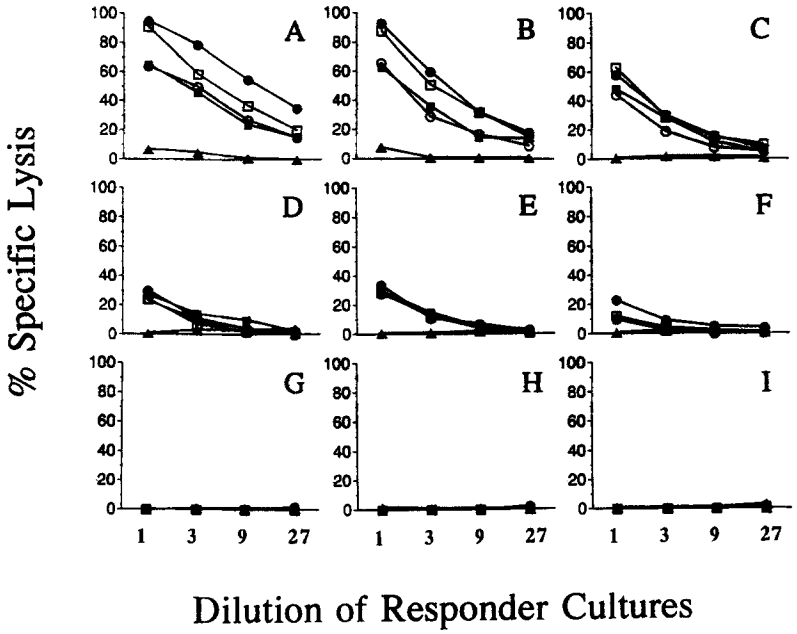
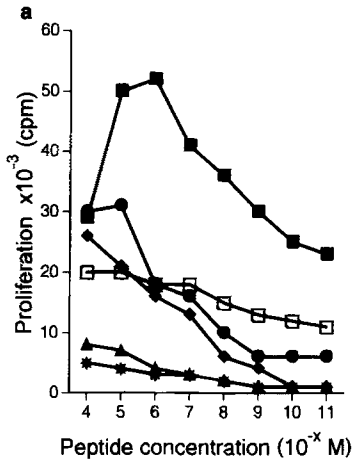


Figure 4. Primary in vitro generation of cytotoxicity demonstrates that the A4Y agonist has a reduced ability to interact with the transgenic TCR. The cytotoxic activity of naive transgenic T cells cultured with p33 (*A–C*), A4Y (*D–F*), or AV (*G–I*) at concentrations of 10^{-6} (*A*, *D*, and *G*), 10^{-8} (*B*, *E*, and *H*), or 10^{-10} M (*C*, *F*, and *I*) were incubated with target cells coated with 10^{-6} or 10^{-8} M (*solid* and *open symbols*, respectively) p33 (■), A4Y (●), or AV (▲).



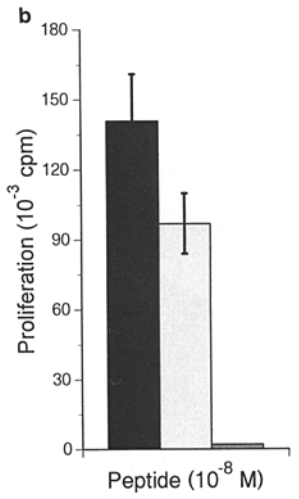


Figure 5. The peptide A4Y can activate mature T cells and induce reduced proliferation relative to p33. (*a*) The proliferative response of mature spleen cells from TCR-transgenic mice was measured when stimulated with macrophages pulsed with various concentrations of p33 (■) or the alanine-substituted variants A1K (▲), A3V (●), A4Y(□), A6F (★), or A8T (◆). Background proliferation for T cells incubated with macrophages coated with AV peptide was 600 cpm. Similar results were obtained when this experiment was repeated three times. (*b*) TCR-specific proliferation was assayed using TCR$^+$ RAG$^{-/-}$ splenocytes stimulated with macrophages that were prepulsed with 10^{-8} M p33 (*solid bar*), A4Y (*white bar*), or AV (*shaded bar*). Background proliferation was 2,000 cpm. Repetition of this experiment produced similar results.

duces a similar range of T cell effector functions as the strong peptide agonist, p33, and that the only noticeable difference is in the magnitude of the response.

A4Y Is Not an Antagonist Peptide. To ensure that A4Y did not have any antagonistic properties, this peptide was tested for its ability to inhibit transgenic T cell proliferation in the presence of the strong agonist peptide, p33. As shown in Fig. 7, the altered peptide S4Y was able to inhibit p33-induced T cell proliferation over a wide range of concentrations in a dose-dependent manner, characteristic of an antagonist peptide. In contrast, A4Y did not inhibit T cell proliferation at any concentration tested. Instead, this peptide displayed a synergistic effect with p33 in a dose-dependent manner. Since A4Y can activate T cells, induce effector functions, and does not demonstrate any properties associated with peptide antagonists, these experiments suggest that A4Y is a moderate agonist.

Different Concentrations of A4Y Induce Positive Selection but Not Clonal Deletion of the Transgenic TCR. To determine whether different concentrations of A4Y could induce positive or negative selection, TCR $\beta_2m^{-/-}$ fetal thymic lobes were cultured over a wide range of peptide concentrations (Fig. 8 *a*). TCR $\beta_2m^{-/-}$ thymic lobes cultured in the presence of 10^{-4}–10^{-9} M A4Y led to efficient maturation of 33% (± 7%, n = 20) CD8$^+$ TCR$^+$ thymocytes. Lower concentrations of A4Y did not affect transgenic TCR$^+$ thymocyte maturation. Interestingly, clonal deletion could not be induced at any concentration using A4Y.

Previous reports have suggested that the density of the MHC molecules on the cell surface contribute to the overall avidity that influences selection events in the thymus (12, 40, 41). Therefore, TCR $\beta_2m^{+/+}$ thymic lobes were cocultured with p33 or A4Y to determine whether clonal deletion could be induced in the presence of "normal" lev-

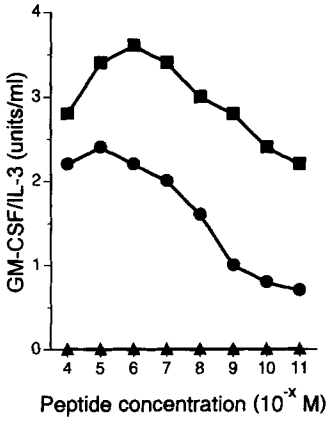


Figure 6. The peptide agonists p33 and A4Y both induce GM-CSF/IL-3 production. GM-CSF/IL-3 production from TCR-transgenic spleen cells stimulated with peptide-coated macrophages was measured after 24 h of cocultivation. The APC were previously pulsed with various concentrations of p33 (■), A4Y (●), or AV (▲). This experiment was repeated twice, producing similar results.

els of class I expression. As expected, $CD8^+$ thymocytes expressed high levels of Vα2 in the absence of exogenous peptide (Fig. 8 *b*). A similar phenotype was observed in the presence of 10^{-6} M A4Y. In contrast, cocultivation in the presence of 10^{-6} M p33 led to reduced fetal thymic lobe cellularity and a few immature $CD8^+$ cells expressing low levels of Vα2. Thus A4Y does not induce efficient clonal deletion of thymocytes expressing the transgenic TCR in $\beta_2m^{+/+}$ thymic lobes.

Functional Specificity of TCR-transgenic Thymocytes Is Altered in the Presence of A4Y. To examine whether the thymocytes that mature in fetal organ culture were tolerant or responsive to the peptide that promoted positive selection, proliferation assays were done. $CD8^+$ thymocytes from TCR β_2m^+ thymic lobes selected in the presence of A4Y and TCR $\beta_2m^{-/-}$ thymic lobes were incubated with APC that were pulsed with p33, A4Y, or AV (Fig. 9). Although TCR β_2m^+ thymocytes proliferated in response to both A4Y and p33, thymocytes positively selected by A4Y could only mount a comparable proliferative response against p33. These results demonstrate that the functional specificity of thymocytes expressing the same TCR is altered depending on the selecting peptide.

Discussion

Properties of Positively Selecting Peptides. Using fetal thymic organ cultures, a variety of different peptides have been directly implicated in thymocyte selection. More specifically, antagonists, partial agonists, and strong peptide agonists have been shown to promote T cell maturation. Because these peptides have strikingly different effects on mature T cells, the significance of these ligands during thymocyte ontogeny has remained controversial. In one instance, a strong agonist was shown to induce both positive and negative selection at different peptide concentrations (15, 16), suggesting that altered TCR–peptide–MHC avidity determined the fate of maturing T cells. On the other hand, it was found that high concentrations of peptide antagonists could induce positive selection (12, 13), implying that an altered TCR–peptide–MHC interaction was required for thymocyte maturation. Current experiments using the peptide A4Y suggest that the correlation between antagonists or partial agonists and positive selection is coincidental. A4Y is clearly an agonist since it can activate naive transgenic T cells and induce T cell effector functions. At the same time, this peptide can positively select thymocytes over a large concentration range, including the high peptide concentrations required for antagonist peptides. Since A4Y induces T cell effector functions and does not demonstrate any properties characteristic of a peptide antagonist, this work shows that agonists are capable of mediating positive selection. More importantly, agonists can promote thymocyte maturation at peptide concentrations that activate mature T cells in vitro.

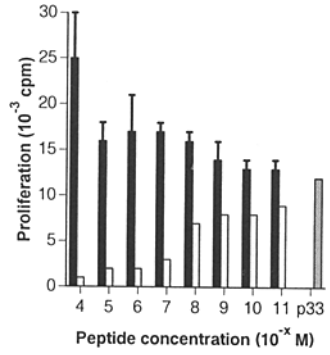


Figure 7. A4Y is not a peptide antagonist for the transgenic TCR. Transgenic T cell proliferation in response to APC prepulsed with 10^{-8} M p33 was assayed in the presence or absence of the indicated peptides. Background proliferation was 1,000 cpm. Five other similar experiments yielded comparable results. (*solid bar*, A4Y; *light shaded bar*, S4Y; *dark shaded bar*, p33).

Thymocyte Tolerance. Our studies have shown that high concentrations of A4Y (10^{-4} M) cannot induce clonal deletion of transgenic T cells in TCR $\beta_2m^{-/-}$ and TCR $\beta_2m^{+/+}$ fetal thymic organ cultures (Fig. 8 and data not shown). However, transgenic T cells that were positively selected in the presence of A4Y were unable to respond against this agonist in proliferation assays, in spite of the fact that these mature T cells could mount a response against the strong peptide agonist, p33. Therefore, these data suggest that transgenic thymocytes are not clonally deleted but rendered tolerant to A4Y.

Interactions in the thymus that lead to clonal unresponsiveness have not been well defined. Models using Mls-1^a suggest that T cells become unresponsive when tolerogen is presented in suboptimal conditions (42, 43), or when T cells are unable to efficiently interact with Mls-1^a (29, 44). These experiments argue that relatively less efficient TCR interactions lead to reduced T cell stimulation and result in unresponsiveness instead of deletion (45). This interpretation is consistent with our findings. Since A4Y cannot induce as strong a proliferative response as p33, this suggests that A4Y cannot engage the transgenic TCR as efficiently, and as a result cannot induce clonal deletion in FTOC.

Previous studies examining interactions that lead to clonal deletion have shown that antigens capable of activating mature T cells can clonally delete these thymocytes during ontogeny (46–49). Further studies have demonstrated that even antigens that are unable to activate peripheral T cells expressing a defined TCR can induce TCR-specific thymocyte clonal deletion (50, 51). In addition, experiments have shown that less antigen is required for clonal deletion

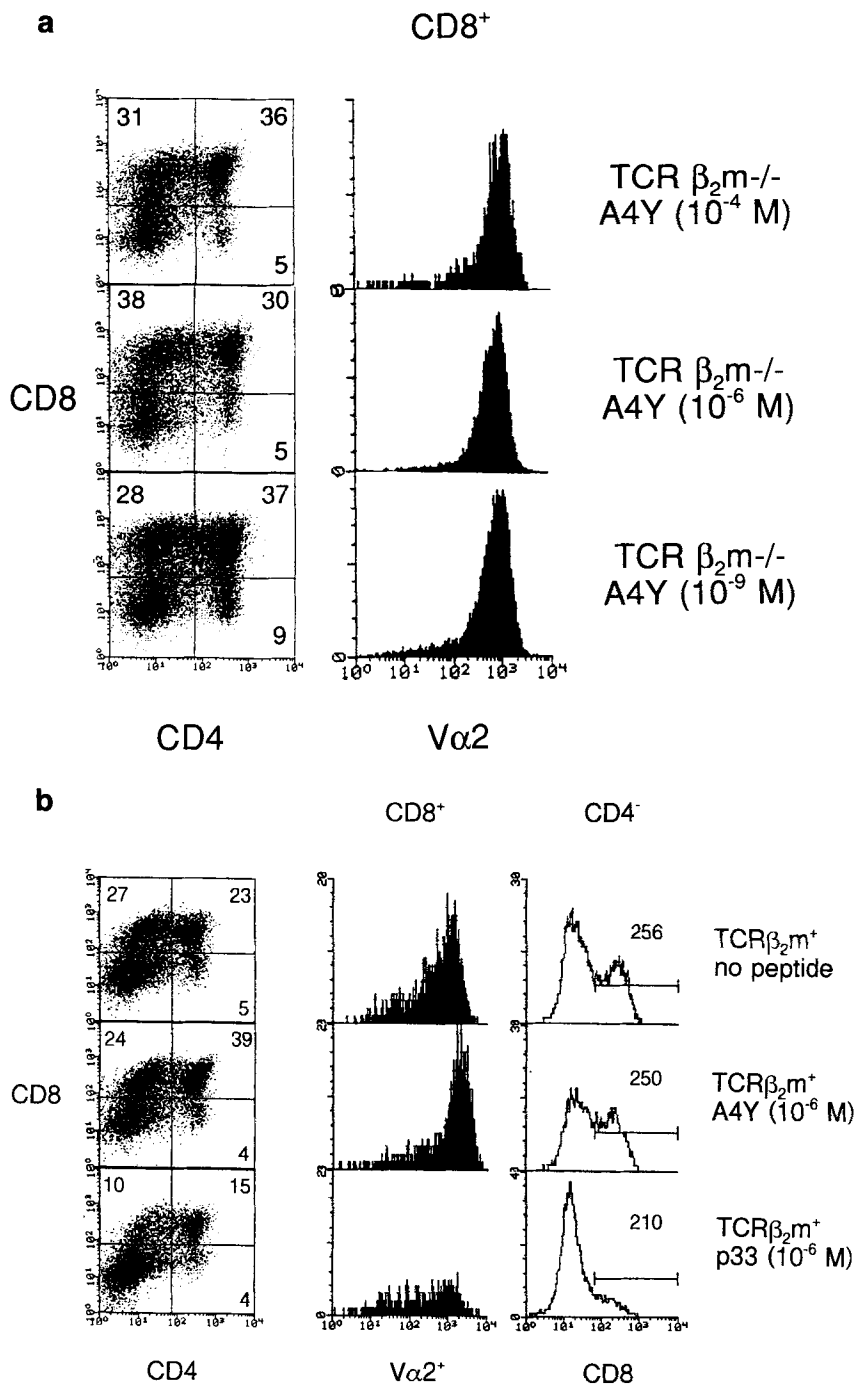


Figure 8. The peptide A4Y induces positive selection but not clonal deletion of TCR$^+$ thymocytes. (*a*) TCR $\beta_2m^{-/-}$ fetal thymocytes were positively selected over a wide range of A4Y peptide concentrations. These thymic lobes were cultured with different concentrations of A4Y and analyzed with antibodies specific for CD4, CD8, and Vα2. The data shown are from 1 experiment representative of 10. (*b*) TCR β_2m^+ fetal thymocytes were cultured in media alone or high concentrations of A4Y or p33. Thymocytes from these lobes were then analyzed with antibodies specific for CD4, CD8, and Vα2. Vα2 profiles of CD8$^+$ cells are shown. After gating for CD4$^-$ thymocytes, CD8 expression was also determined. The mean fluorescence of the CD4$^-$CD8$^+$-gated cells is displayed. Similar results were obtained when this experiment was repeated five times.

than T cell activation (52, 53). One interpretation of these studies has been that the requisite interactions for clonal deletion of thymocytes are less stringent than those required for T cell activation. This hypothesis is attractive because it ensures that T cells with potential self-reactivity are eliminated during ontogeny, and consequently autoimmunity is avoided in the periphery.

Nonetheless, our results conflict with this view of thymocyte tolerance. Although A4Y can stimulate naive transgenic T cell effector functions, this peptide does not induce clonal deletion when cocultured with TCR $\beta_2m^{-/-}$ or TCR $\beta_2m^{+/+}$ thymic lobes. It is possible that the avidity of thymocyte–stromal cell interactions dictates the form of negative selection imposed on the developing T cell. High avidity interactions lead to clonal deletion, whereas lower avidity interactions result in unresponsiveness. In this case, interactions with A4Y do not surpass an avidity required for clonal deletion. Instead, the transgenic thymocytes are positively selected and modified by the selecting ligand so that they no longer respond against A4Y.

Previous studies have shown that coreceptor downregulation may be one of the mechanisms used by thymocytes

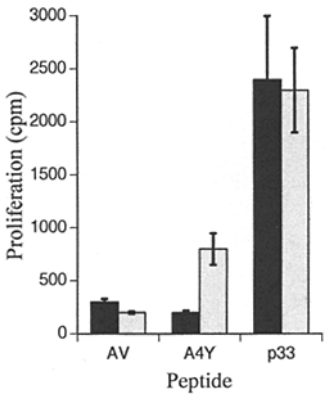


Figure 9. TCR$^+$ thymocytes selected in the presence of A4Y are tolerant to the positively selecting ligand but proliferate in response to p33. TCR$^+$ CD8$^+$ thymocytes from TCR $\beta_2m^{-/-}$ thymic lobes cultured with A4Y (*solid bars*) or TCR β_2m^+ thymic lobes cultured in media alone (*shaded bars*) were cocultured with peptide-coated APC. Background proliferation for TCR T cells cultured with macrophages in the absence of peptide was 200 cpm. These results are representative of five experiments.

to compensate for high affinity TCR interactions (13, 54, 55), resulting in unresponsiveness instead of clonal deletion. In our present experiments, analysis of TCR$^+$CD8$^+$ thymocytes maturing in the presence of A4Y did not show altered CD8 levels when compared with T cells from TCR β_2m^+ thymic lobes (Fig. 8 *b*). However, it is possible that other molecules contributing to the overall avidity of T cell interactions have been altered.

Alternatively, thymocyte selection may induce a biochemical change in the developing T cell, resulting in unresponsiveness to defined ligands. Our findings suggest that peptides capable of mediating positive selection imprint a "resting threshold" or a "resting state" on these thymocytes. As a consequence, the T cells become unresponsive to further encounters with the positively selecting peptide. Thymocyte modification during positive selection establishes a mechanism for preventing peripheral autoimmunity. Previous experiments have shown that thymic epithelium is capable of mediating positive (56) and negative selection (57–60). Therefore, it is possible that positive selection and thymocyte modification leading to unresponsiveness both occur during interactions with the thymic epithelium. However, in our model system it is not possible to determine whether tolerance to A4Y is imprinted by the thymic epithelium or during further interactions with bone marrow–derived cells.

A recent study addressed the role of agonists during thymocyte maturation by using an ovalbumin peptide and strong agonist variants of this epitope in TCR $\beta_2m^{-/-}$ FTOC (14). Although the strong agonist variant could stimulate TCR$^+$ CD8$^+$ phenotypic maturation, these T cells could not respond against ovalbumin as measured by proliferation assays. For this reason, the authors concluded that agonist peptides cannot mediate positive selection of functional CD8$^+$ T cells. However, the present work demonstrates that agonists can indeed produce mature CD8$^+$ T cells and that these cells are functional. In our model, mature thymocytes are rendered tolerant toward positively selecting ligands, and they are predicted to respond against undefined, higher affinity peptides (29). This prediction is supported by recent experiments that have demonstrated that a given T cell receptor can respond against a variety of epitopes that contain unrelated peptide sequences (61, 62). This suggests that high affinity peptides may be totally unrelated to the positively selecting ligand. In the case of ovalbumin variants that possess strong agonist properties, it is possible that assays testing TCR$^+$CD8$^+$ T cell function have not used peptides exhibiting high enough affinity to surpass the defined resting threshold. We believe that a subset of agonist peptides can induce thymocyte differentiation and functional reactivity while maintaining tolerance to selecting peptides.

Models of Thymocyte Selection. Models of thymocyte development have to explain how a TCR can discriminate between a positively and negatively selecting ligand. In addition, these hypotheses must incorporate current data demonstrating the contribution of peptides during these events. To this extent, recent experiments have favored an efficacy model of T cell maturation (18). In this case, efficacy is defined as the ability of a ligand to catalyze TCR-mediated biological activity. According to this model, a certain TCR–peptide–MHC affinity threshold must be surpassed in order for a thymocyte to be eligible for positive selection. Ligands that meet these criteria can potentially determine whether positive or negative selection occurs. Peptides that are unable to initiate T cell activation are predicted to promote positive selection. In contrast, peptides that are capable of provoking receptor-mediated activity (efficacy) induce negative selection. If this model is correct, then antagonist peptides, which according to this hypothesis have no efficacy, should positively select, and peptide agonists, which have high efficacy, should negatively select maturing T cells. Studies by Hogquist et al. (12, 13) and Jameson et al. (14) support this hypothesis. However, current experiments using A4Y demonstrates that an agonist capable of inducing TCR-mediated activity can positively select thymocytes. These findings conflict with a simple efficacy model of thymocyte selection.

A related developmental model postulates that positive selection is the result of incomplete TCR signaling (3, 22). This hypothesis proposes that extensive TCR–peptide–MHC cross-linking without suitable conformational change generates incomplete signaling and lack of T cell activation. Antagonist peptides have been postulated to interact with T cells in such a manner and induce positive selection. In addition, induction of conformational change without extensive cross-linking would provide too weak a signal to activate thymocytes, thus providing an explanation as to how low concentrations of agonist peptides such as p33 positively select. Frequent binding of activating agonist peptides would result in clonal deletion. However, current experiments are not consistent with this model. Instead, high concentrations of A4Y, which should induce an agonist-like conformational change, positively selects transgenic thymocytes. Therefore, a conformational model that relies on incomplete TCR signaling to induce positive selection cannot explain current results.

Alternatively, these data may be interpreted in terms of an affinity/avidity model of T cell maturation (17). Like the efficacy hypothesis, this model assumes that a thymocyte must have adequate avidity for peptide–MHC to be posi-

tively selected. However, an affinity/avidity model does not limit positively selecting ligands to antagonist or partial agonist peptides. For instance, different concentrations of A4Y, ranging from 10^{-9} to 10^{-4} M, can interact with transgenic TCRs and provide sufficient stimulation to induce positive selection. However, if the only role of a positively selecting peptide is to contribute to TCR–peptide–MHC affinity, then other peptides with the ability to induce similar T cell responses should promote similar levels of thymocyte maturation. This does not seem to be the case. Alanine-substituted variants of the wild-type peptide that produce comparable levels of proliferation relative to A4Y (Fig. 5 *a*) were not nearly as efficient as A4Y at promoting transgenic T cell maturation. Although the affinity between the TCR and ligands has not been directly measured, our results suggest that A4Y has an undefined, intrinsic capacity to positively select transgenic thymocytes that cannot be fully explained using an affinity/avidity model.

If positively selecting peptides have an intrinsic quality, then current experiments demonstrate that this attribute is not confined to antagonists or partial agonists. There is no direct correlation between a peptide's ability to positively select thymocytes and its ability to activate mature T cells. Instead, our data suggest that positively selecting ligands are made up of a distinct subset of peptides that are able to generate unique interactions with the TCR. Peptides that meet a basic requirement, be it a distinct conformational change or a novel form of TCR stimulation, positively select thymocytes. In addition, experiments using various concentrations of the high affinity peptide p33 show that there must be some signaling gradient that directly or indirectly reflects thymocyte–stromal cell avidity. For this reason, we propose that peptides that are capable of unique TCR interactions induce positive selection. Thymocytes that are positively selected by these peptides are modified such that they remain tolerant against these ligands. This "resting threshold" may also be altered during intermediate avidity interactions that are independent of positive selection (29). However to maintain this resting threshold, continual TCR-peptide/MHC interactions may be required. Higher avidity interactions push these thymocytes toward clonal deletion. As a result, mature T cells that have undergone this selection process are unresponsive to self-peptides encountered during development and yet are capable of responding against higher affinity foreign peptides in the periphery.

Although previous models have incorporated ideas of dynamic T cell reactivity and variable activation thresholds (63, 64), these concepts have not been supported by solid experimental evidence. Our results suggest that a T cell's "resting state" is fine tuned during positive and negative selection events in the thymus. It remains to be determined whether this process involves modification of thymocyte avidity or modulation of an intrinsic biochemical pathway.

We thank D. Bouchard for cell sorting and J.C. Zúñiga-Pflücker, V.A. Wallace, J. Penninger, and J.R. Carlyle for critically reading the manuscript.

This work was supported by the Medical Research Council (MRC) of Canada. P.S. Ohashi is a recipient of an MRC Scholarship.

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Received for publication 5 September 1995 and in revised form 8 November 1995.

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