

# Positive Selection of T Cells: Rescue from Programmed Cell Death and Differentiation Require Continual Engagement of the T Cell Receptor

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## Summary

Positive selection of T cells is a complex developmental process generating long-lived, functionally mature CD4<sup>+</sup>CD8<sup>-</sup> and CD4<sup>-</sup>CD8<sup>+</sup> cells from short-lived, immature CD4<sup>+</sup>CD8<sup>+</sup> precursors. The process is initiated in the thymus by interaction of the  $\alpha\beta$  TCR with molecules encoded by the MHC, occurs without cell division, and involves rescue from programmed cell death (PCD), as well as induction of differentiation and maturation of selected precursors. It is unclear whether development of small, positively selected CD4<sup>+</sup>CD8<sup>+</sup> thymocytes (characterized by up-regulated levels of TCR and CD69 molecules) depends on further interactions with MHC molecules and, if so, whether such interactions are required for survival, for maturation, or for both. The involvement of the TCR and/or CD4/CD8 coreceptors in transmitting additional signals is also unknown. We have examined these questions by analyzing survival and differentiation of early (CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>hi</sup>) and later (CD4<sup>-</sup>CD8<sup>+</sup>TCR<sup>hi</sup>) postselection stages of thymocytes from normal and bcl-2 transgenic mice expressing transgenic, class I MHC-restricted TCR, upon intrathymic transfer into recipients that lacked ligands either for both the TCR and CD8 coreceptor, or for the TCR only. The results provide direct evidence that induction of differentiation of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes by recognition of MHC molecules does not rescue them from PCD and is insufficient to activate the entire maturation program. Both processes require continual engagement of the TCR by positively selecting MHC molecules that, at least in the case of class I MHC-restricted CD4<sup>-</sup>CD8<sup>+</sup> T cells, cannot be substituted by the engagement of coreceptor alone.

According to the concept of programmed cell death (PCD)<sup>1</sup> (reviewed in reference 1), survival and development of cells depend on suppression of apoptosis, which can be viewed as a biochemical "time bomb" that will kill the cell at a predetermined hour unless its clock is set back by environmental factors. This view poses an interesting problem concerning the relationship between signals that rescue cells from PCD and those that induce differentiation and/or maturation at various developmental stages of different cell lineages. Here we are concerned with this question in relation to the role of the  $\alpha\beta$  TCR and CD8 coreceptor-mediated interactions with class I MHC molecules during T cell development. These interactions are believed to be critically involved in the generation of the majority of long lived (>8 wk), mature CD4<sup>-</sup>CD8<sup>+</sup> (CD8<sup>+</sup>) T cells recognizing antigenic peptides presented by class I MHC molecules from short lived (3.5 d), small, resting CD4<sup>+</sup>CD8<sup>+</sup> thymocytes.

This process and the parallel process of generation of the majority of CD4<sup>+</sup>CD8<sup>-</sup> (CD4<sup>+</sup>) T cells, which involves TCR and CD4 coreceptor-mediated interactions with class II MHC molecules, are called positive selection (for a recent review, see reference 2).

Positive selection of mature T cells from CD4<sup>+</sup>CD8<sup>+</sup> precursors does not require cell division (3) and is accompanied by a number of changes in addition to down-modulation of CD4 or CD8 molecules. They include the up-regulation of cell surface expression of the TCR (4, 5), an increase of Bcl-2 protein levels (6, 7), termination of activity of recombination-activating genes (RAG-1 and RAG-2) (8, 9), transient expression of an early activation marker CD69 (10, 11), and down-regulation of heat-stable antigen (HSA) expression (11, 12).

The role of TCR- and/or CD4/CD8 coreceptor-mediated interactions in the rescue from PCD and in the induction of differentiation of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes is unclear and has only recently begun to be analyzed in more detail. It was shown that Bcl-2-mediated suppression of apoptosis in the absence of TCR-ligand interaction does not result in any of

<sup>1</sup> Abbreviations used in this paper: APC, allophycocyanin;  $\beta$ 2m,  $\beta$ 2-microglobulin; HSA, heat-stable antigen; PCD, programmed cell death.

the phenotypic changes accompanying positive selection (7, 13), indicating that TCR-mediated signals are required to initiate the differentiation of CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>lo</sup> thymocytes and that interactions with MHC molecules via CD4/CD8 coreceptors alone are unable to do so, even when CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>lo</sup> thymocytes are rescued from PCD by the bcl-2 transgene. However, the requirement for interactions with positively selecting MHC-peptide complexes in suppressing apoptosis and their role in promoting further differentiation of selected CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>hi</sup> thymocytes that have already received a TCR-mediated signal have not been directly analyzed.

Several recent studies provided indirect evidence suggesting that the initial TCR-ligand interaction resulting in up-regulation of cell surface TCR levels and down-regulation of CD4 or CD8 coreceptors may not be sufficient to rescue immature thymocytes from PCD and that more than a single interaction of developing T cells with positively selecting MHC-peptide complexes may be necessary for end-stage differentiation (11, 14–19). For example, detection of thymocytes arrested at the CD4<sup>+</sup>CD8<sup>lo</sup>TCR<sup>hi</sup>HSA<sup>hi</sup> stage of differentiation in class II MHC-deficient mice (14, 20) and an analogous population of CD4<sup>lo</sup>CD8<sup>+</sup>TCR<sup>hi</sup>HSA<sup>hi</sup> thymocytes in class I MHC-deficient mice (15, 16) was interpreted as indicating that CD4<sup>+</sup>CD8<sup>+</sup> thymocytes engage positively selecting MHC-peptide complexes twice en route to becoming end-stage CD4<sup>+</sup> and CD8<sup>+</sup> thymocytes: the first engagement would provoke random down-modulation of either CD4 or CD8 molecules; the second (requiring participation of the appropriate coreceptor) would permit end-stage differentiation (14). The successful differentiation of functionally mature CD8<sup>+</sup> (endogenous CD4<sup>-</sup>) T cells in class I MHC-deficient mice expressing the CD4 transgene (15, 21) and CD4<sup>+</sup> T cells in class II MHC-deficient mice expressing the CD8 transgene (22) seems to support this interpretation, but whether involvement of the TCR in delivering the second signal was required or whether coreceptor-mediated interactions with MHC molecules without participation of the TCR were sufficient remains an open question. This question becomes relevant and important in view of the results of a recent study (18), in which it was found that CD4<sup>-</sup>CD8<sup>+</sup>TCR<sup>hi</sup>HSA<sup>hi</sup> thymocytes in rearrangement-deficient mice expressing only a single species of transgenic TCR, restricted by class II MHC molecules, could not fully mature if selected in class I MHC-deficient mice even though the TCR showed no detectable cross-reactivity with class I MHC molecules. This result was interpreted as consistent with the notion that single interaction with positively selecting MHC-peptide complexes is not sufficient to induce end-stage differentiation of developing thymocytes, thus implying that a second interaction (in this case of CD8 with class I MHC molecules not involving coengagement of the TCR) was probably involved.

All of these observations stem from the analysis of thymocytes expressing “mismatched” combinations of the TCR with CD4/CD8 coreceptors, and one cannot be sure whether the apparent failure to rescue these cells from PCD and the postu-

lated requirement for multiple TCR-mediated interactions apply as well to CD4<sup>+</sup>CD8<sup>+</sup> thymocytes, which down-modulated the “matched” coreceptor as a result of the first encounter of the TCR with positively selecting ligand. Thus, the following specific questions concerning the role of TCR- and CD4/CD8 coreceptor-mediated interactions in intrathymic T cell development remain unanswered: (a) Is the development of small CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>hi</sup> thymocytes that were activated by the initial TCR-mediated signal dependent on or independent from further interactions of the TCR with its ligand? (b) Is the development dependent on interactions involving coreceptors alone? (c) If the development of selected CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>hi</sup> thymocytes is dependent on further interactions with positively selecting ligands, are they required for survival or for maturation, or for both?

Here we directly examine these questions by analyzing survival and differentiation of isolated early and late postselection stages of thymocytes expressing transgenic, class I MHC-restricted TCR upon intrathymic transfer into recipients that lack ligands either for both the TCR and CD8 coreceptor, or for the TCR only.

## Materials and Methods

**Mice.** C57BL/6 (B6), BALB/c, and (B6 × BALB/c)F1 (BDF1) mice were obtained from the animal colony of the Basel Institute for Immunology (Basel, Switzerland). AKR/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The H-2<sup>b</sup> and H-2<sup>d</sup> TCR $\alpha\beta$  transgenic mice expressing receptor specific for the HY antigen in the context of H-2D<sup>b</sup> molecules were described previously (23, 24) and were bred at the Basel Institute for Immunology. The H-2<sup>b/b</sup> and H-2<sup>d/d</sup> bcl-2/TCR bitransgenic mice were produced by serial crosses of the transgenic E $\mu$ -bcl-2-25 strain (24), kindly provided by Dr. S. Cory (Walter and Eliza Hall Institute of Medical Research, Royal Melbourne Hospital, Victoria, Australia), with the H-2<sup>b/b</sup> or H-2<sup>d/d</sup> anti-HY TCR $\alpha\beta$  transgenic mice. The bone marrow irradiation chimeras were produced by intravenous injection of T cell depleted (anti-Thy1.2 + C treated, 45 min, 37°C) bone marrow cells ( $3 \times 10^6$  per mouse) from H-2<sup>d/d</sup> bcl-2/TCR bitransgenic donors into lethally irradiated (950 rads) H-2<sup>b/d</sup> BDF1 mice. The chimeric mice served as donors of thymocytes 8–10 wk after irradiation and bone marrow reconstitution. All other mice were used in experiments at the age of 6–10 wk.

**Antibodies and Other Reagents.** The following conjugates of mAbs were used: T3.70-FITC (anti-HY/D<sup>b</sup> transgenic TCR) (23), anti-CD4-PE (Becton Dickinson, Heidelberg, FRG), anti-CD8-biotin (Becton Dickinson), anti-CD8-Red-613 (GIBCO BRL, Gaithersburg, MD), M1/69-biotin (anti-HSA) (25), and anti-Thy1.2-FITC (Becton Dickinson). FITC-conjugated Fab' fragments of mAb T3.70 were kindly provided by Jorg Kirberg (Basel Institute of Immunology). Streptavidin-allophycocyanin (APC) conjugate was purchased from Southern Biotechnology Associates (Birmingham, AL), and streptavidin-Tri-Color conjugate was purchased from Caltag Laboratories (San Francisco, CA).

**Sorting of Thymocytes.** The small CD4<sup>+</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> and small CD4<sup>-</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> cells (Fig. 1) were sorted at 4°C by FACStar-Plus (Becton Dickinson) flow cytometer as described (11, 26) from the populations of thymocytes, which were triple stained with anti-CD4-PE, anti-CD8-Red-613, and Fab' fragments of T3.70-FITC mAbs. Fab fragments of mAb T3.70 were used to avoid activation of sorted cells. In some experiments anti-CD8-

biotin followed by streptavidin-Tri-Color was used instead of Red-613-conjugated anti-CD8 mAb.

**Intrathymic Transfer and Fluorometric Analysis of the Fate of Injected Cells.** Sorted populations of cells were injected into both thymic lobes of nonirradiated, anesthetized recipients ( $1-3 \times 10^5$  cells per lobe in  $10\text{-}\mu\text{l}$  volumes) with the aid of a B-D Micro-Fine IV syringe mounted on a TRIDAK Stepper (Indicon Inc., Brookfield Center, CT).

2-4 d later, the mice were sacrificed, and their thymuses were removed and analyzed for the presence of the injected cells as follows. Thymocyte suspension from the whole thymus was prepared in PBS supplemented with 2% FCS, and total cell number was counted before the first wash. After washing and removing clumps of aggregated dead cells, the remaining cells were stained with T3.70-FITC, anti-CD4-PE, anti-CD8-Red-613, and M1/69-biotin plus streptavidin-APC or with T3.70-FITC, anti-CD4-PE, anti-CD8-biotin plus streptavidin-Tri-Color and analyzed on FACStar-Plus flow cytometer. In some experiments thymocyte suspension was divided into two: one half were stained with T3.70-FITC, anti-CD4-PE, and anti-CD8-biotin plus streptavidin-Tri-Color; the other half were stained with T3.70-FITC and M1/69-biotin plus streptavidin-Tri-Color; alternatively, one half were stained with T3.70-FITC, and the other half were stained with anti-Thy1.2-FITC. Stained cells were analyzed on FACScan flow cytometer. 5-10 million thymocytes were analyzed by cytofluorometer, but acquisition gates were set such as to exclude the great majority of FITC-negative cells from further analysis. The live cells were then gated from acquired FITC-positive cells on the basis of forward and side scatter profile to reveal the population of T3.70<sup>+</sup> or Thy1.2<sup>+</sup> cells. Analysis of the size of recovered T3.70<sup>hi</sup> cells revealed that they consisted exclusively of small cells. The analysis of CD4, CD8, and HSA expression was performed on gated live T3.70<sup>hi</sup> cells.

The total number of recovered T3.70<sup>hi</sup> cells was calculated as follows: (total no. of thymocytes) (no. of T3.70<sup>hi</sup> cells in analyzed sample)/(no. of cells analyzed). The percent recovery of T3.70<sup>hi</sup> cells was calculated as follows: (total no. of recovered T3.70<sup>hi</sup> cells) (100)/(no. of injected T3.70<sup>hi</sup> cells).

## Results

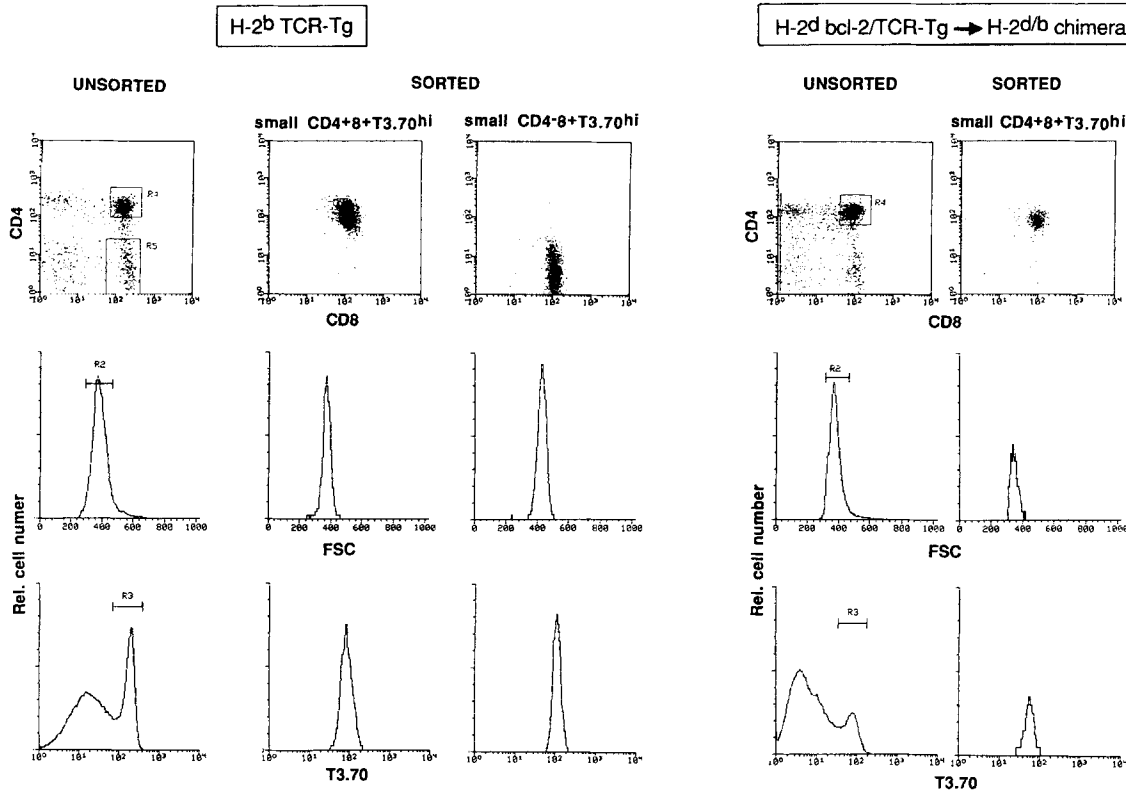
**Experimental Approach.** To obtain insight into the role of interactions with positively selecting ligands in rescue from PCD and in differentiation of developing T cells, we followed the fate of purified populations of small TCR<sup>hi</sup> thymocytes (CD4<sup>+</sup>CD8<sup>+</sup> or CD4<sup>-</sup>CD8<sup>+</sup>; see Fig. 1) expressing a transgenic, class I MHC (H-2D<sup>b</sup>)-restricted, male (HY) antigen-specific TCR (T3.70<sup>+</sup>) (23, 24), after intrathymic transfer into three groups of recipients: (a) normal syngeneic (H-2<sup>b</sup>) female mice expressing the positively selecting ligand with which both the TCR and CD8 coreceptor could interact; (b) syngeneic (H-2<sup>b</sup>), class I MHC-deficient ( $\beta 2$ -microglobulin<sup>-/-</sup> [ $\beta 2m^{-/-}$ ]) female mice lacking ligands for both the TCR and CD8 coreceptor; and (c) normal allogeneic (H-2<sup>d</sup> or H-2<sup>k</sup>) female mice lacking the ligand for the TCR but not for CD4/CD8 coreceptors. The injected small CD4<sup>+</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> thymocytes represented cells that already received at least one positive selection signal because, as previously demonstrated (11, 27), they express elevated levels of CD69 molecules and can be found in mice expressing positively selecting H-2D<sup>b</sup> molecules and not in mice lacking

these molecules. We reasoned that analysis of the survival and differentiation of purified CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>hi</sup> thymocytes from TCR transgenic or bcl-2/TCR double transgenic donors, injected intrathymically into these mice, should not only establish whether further interactions with positively selecting ligands are necessary, but should also distinguish the developmental requirement for engagement of the TCR from requirement for engagement of coreceptor molecules alone (if, as suggested by experiments of Kirberg et al. (18), the latter type of interactions play a role in development of immature T cells).

We showed previously (28) that the differentiation of small CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>lo</sup> thymocytes into mature CD4<sup>-</sup>CD8<sup>+</sup>TCR<sup>hi</sup> cells via the CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>hi</sup> stage can proceed in the thymus of allogeneic, H-2-incompatible recipients expressing selecting class I MHC molecules. This indicated that an alloresponse against intrathymically injected cells either did not take place or was insufficient to interfere with positive selection and that anti-CD4, anti-CD8, and anti-TCR antibodies used to purify the injected cells (see Materials and Methods) did not adversely affect their ability to differentiate. For these and other reasons that will be discussed later, we believe that it is unlikely that the experimental conditions of our study could unphysiologically interfere with the normal development of injected cells; nevertheless, one should be aware of the possible impact of steroid-mediated stress and generally low cell recoveries in this type of experiments.

**Rescue of Immature Thymocytes from PCD Depends on Continual Engagement of the TCR by Positively Selecting Ligand.** highly purified, small CD4<sup>+</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> thymocytes from H-2<sup>b</sup> TCR transgenic females were injected intrathymically into B6 (H-2<sup>b</sup>) and into class I MHC-deficient  $\beta 2m^{-/-}$  (H-2<sup>b</sup>) as well as into BALB/c (H-2<sup>d</sup>) or AKR/J (H-2<sup>k</sup>) female mice. After 2 or 4 d, the thymuses of recipient mice were analyzed for the presence of injected T3.70<sup>hi</sup> cells, and their CD4/CD8 phenotype was determined. As shown in Fig. 2 and in Table 1, a substantial proportion of injected cells (3-10%) could be recovered from B6 (D<sup>b</sup>) recipients, and >90% of recovered cells were CD4<sup>-</sup>CD8<sup>+</sup>. In contrast, already after 2 d, no or very few (<0.5%) T3.70<sup>hi</sup> cells could be recovered from D<sup>b-</sup> (i.e.,  $\beta 2m^{-/-}$ , BALB/c, or AKR/J) recipients. It is not clear whether the few cells that scored as T3.70<sup>hi</sup> were of the donor origin or represented the "background" of nonspecifically stained cells of the recipient, but in contrast to truly T3.70<sup>hi</sup> cells recovered from the B6 recipient, the great majority of them were CD4<sup>+</sup>CD8<sup>+</sup>. Contrary to CD4<sup>+</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> thymocytes, comparable numbers of the injected CD4<sup>-</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> thymocytes could be recovered from B6 as well as from  $\beta 2m^{-/-}$ , BALB/c, and AKR/J mice (Fig. 3 and Table 1). These results suggested that the CD4<sup>+</sup>CD8<sup>+</sup> cells, in contrast to the CD4<sup>-</sup>CD8<sup>+</sup> cells, did not survive in the absence of TCR engagement by positively selecting ligand and that possible interactions with MHC molecules mediated by coreceptor molecules alone were unable to rescue CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>hi</sup> cells from PCD. However, it should be noted that CD4<sup>-</sup>CD8<sup>+</sup> cells recovered from H-2D<sup>b-</sup> thymuses injected

THYMOCYTES



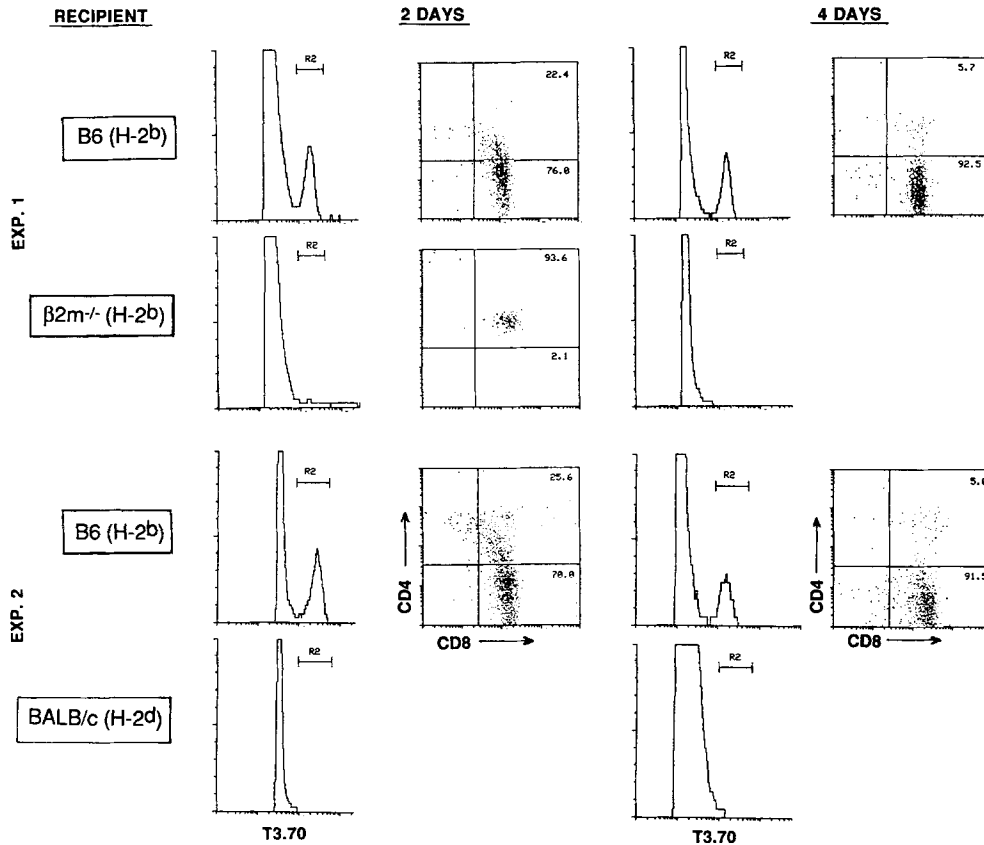
**Figure 1.** Representative phenotypes of purified cell populations used in intrathymic transfer experiments. Small CD4<sup>+</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> and small CD4<sup>-</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> cells were sorted from total populations of thymocytes of the TCR transgenic H-2<sup>b</sup> (or bcl-2/TCR bitransgenic H-2<sup>b</sup>; not shown) and of bcl-2/TCR bitransgenic H-2<sup>d</sup> → H-2<sup>d/b</sup> female mice using the indicated gates as described in Materials and Methods. The purity of sorted populations ranged from 99.3 to 99.8% for TCR transgenic and bcl-2/TCR bitransgenic H-2<sup>b</sup> mice and from 98.2 to 99.1% for bcl-2/TCR bitransgenic H-2<sup>d</sup> → H-2<sup>d/b</sup> chimeric mice. Note that all sorted and reanalyzed populations (immature CD4<sup>+</sup>CD8<sup>+</sup> and mature CD4<sup>-</sup>CD8<sup>+</sup>) exhibited somewhat reduced intensity of staining with all fluorochrome-coupled mAbs (anti-CD4, anti-CD8, and T3.70) as compared with the original staining indicated by gates in unsorted populations. This reflects the general loss of fluorescence accompanying the sorting procedure rather than contamination with cells expressing lower levels of markers in the original population. The possibility that sorted CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>hi</sup> cells contained few unselected CD<sup>+</sup>CD8<sup>+</sup> cells that nonspecifically up-regulated TCR level in vitro (34) cannot be excluded, but it is unlikely in view of the fact that sorting was performed at 4°C (see Materials and Methods).

with CD4<sup>-</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> cells expressed reduced TCR levels as compared with cells recovered from thymuses of B6 (D<sup>b+</sup>) recipients (Fig. 3). Thus, one could argue that the inability to detect T3.70<sup>hi</sup> cells in H-2D<sup>b-</sup> recipients after injection of CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>hi</sup> thymocytes is due to down-modulation of the TCR rather than to the absence of injected cells. To test this possibility, we have used anti-Thy1.2 antibody in addition to mAb T3.70, to search for the presence of injected CD4<sup>+</sup>CD8<sup>+</sup> thymocytes in AKR/J (Thy1.1) recipients. The results shown in Fig. 4 demonstrate that in contrast to the recipients of CD4<sup>-</sup>CD8<sup>+</sup>TCR<sup>hi</sup> cells, no Thy1.2<sup>+</sup> cells could be recovered from the recipient of CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>hi</sup> thymocytes, thus indicating that the inability to detect T3.70<sup>hi</sup> cells results from their true absence and is not due to down-modulation of the TCR. We conclude from these results that recognition of positively selecting ligand by CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>lo</sup> thymocytes leading to up-regulation of TCRs level does not rescue them from PCD

and that survival of selected CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>hi</sup> cells, in contrast to CD4<sup>-</sup>CD8<sup>+</sup>TCR<sup>hi</sup> thymocytes, is dependent on continual interaction of the TCR with positively selecting ligand, which at least in the case of class I MHC-restricted CD8<sup>+</sup> T cells cannot be substituted by interactions mediated by coreceptors alone.

*Not Only Rescue from PCD but Also Differentiation and Maturation of Thymocytes Depend on Continual Engagement of the TCR by Positively Selecting Ligand.* The results previously described do not answer the question of whether multiple engagements of the TCR by positively selecting ligand during the development of mature CD8<sup>+</sup> T cells from CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>hi</sup> thymocytes are needed only to suppress apoptosis or are also needed to induce end-stage differentiation of these cells. To answer this question and to discriminate between the possible requirement for TCR-mediated and TCR-independent coreceptor-mediated induction of end-stage differentiation, we performed similar intrathymic transfer ex-

RECOVERY AND CD4/CD8 PHENOTYPE OF T3.70<sup>hi</sup> CELLS AFTER:

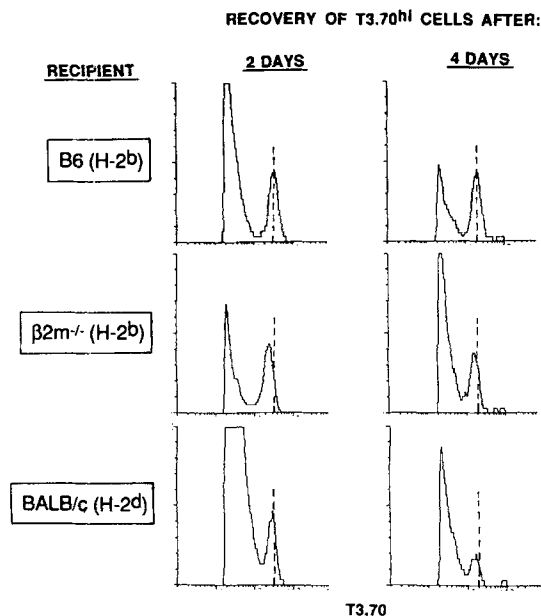


**Figure 2.** TCR transgenic CD4<sup>+</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> thymocytes cannot be recovered from H-2D<sup>b</sup>-deficient recipients. Small CD4<sup>+</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> thymocytes (EXP. 1: 3 × 10<sup>5</sup>, purity 99.6%; EXP. 2: 2 × 10<sup>5</sup>, purity 99.3%) from H-2<sup>b</sup> TCR transgenic female mice were injected intrathymically into the indicated female recipients. 2 or 4 d later, the recipient thymuses were analyzed for the presence of T3.70<sup>hi</sup> cells (gate R2), and their CD4/CD8 phenotypes were determined as described in Materials and Methods. Numbers in quadrants indicate percentages. For percent recovery of T3.70<sup>hi</sup> cells, see Table 1.

**Table 1.** Recovery of Intrathymically Injected Cells (Summary of All Experiments)

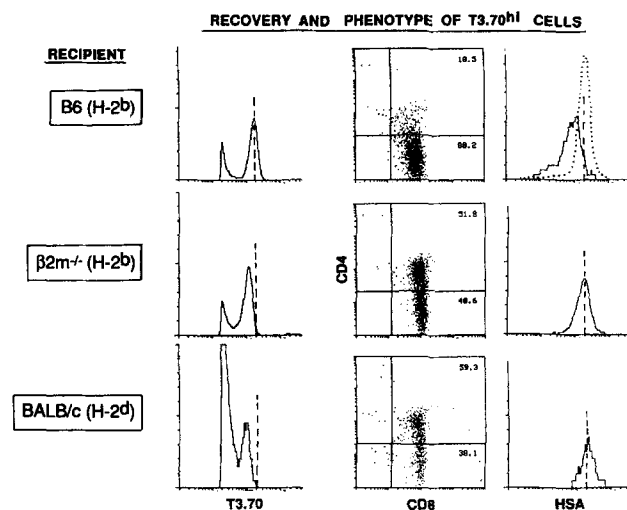
Donor/phenotype of injected cells	Recipient	Recovery (% ± SD)*		
		Day 2	Day 4	Positive/total
H-2 <sup>b</sup> TCR Tg/ CD4 <sup>+</sup> 8 <sup>+</sup> T3.70 <sup>hi</sup>	B6	5.0 (2.4–7.4) (n = 3)	7.1 ± 2.7 (n = 7)	10/10
	β2m <sup>-/-</sup>	<0.5 (n = 1)	<0.2 (n = 3)	0/4
	BALB/c	<0.3 (n = 3)	<0.5 (n = 6)	0/9
	AKR/J	<0.1 (n = 1)	<0.2 (n = 2)	0/3
H-2 <sup>b</sup> TCR Tg/ CD4 <sup>-</sup> CD8 <sup>+</sup> T3.70 <sup>hi</sup>	B6	10.0 (n = 1)	13.9 (11.4–16.4) (n = 2)	3/3
	β2m <sup>-/-</sup>	6.8 (n = 1)	4.3 ± 2.9 (n = 4)	5/5
	BALB/c	11.1 (7.4–14.8) (n = 2)	7.5 ± 2.1 (n = 4)	6/6
	AKR/J	8.0 (n = 1)	6.3 (3.2–9.4) (n = 2)	3/3
H-2 <sup>b</sup> bcl-2/TCR Tg/ CD4 <sup>+</sup> CD8 <sup>+</sup> T3.70 <sup>hi</sup>	B6	ND	15.7 ± 5.6 (n = 4)	4/4
	β2m <sup>-/-</sup>	ND	21.7 (17.1–26.3) (n = 2)	2/2
	BALB/c	ND	17.4 ± 6.2 (n = 4)	4/4
H-2 <sup>d</sup> bcl-2/TCR Tg/ CD4 <sup>+</sup> CD8 <sup>+</sup> T3.70 <sup>hi</sup>	B6	ND	10.8 (8.2–13.0) (n = 3)	3/3
	BALB/c	ND	8.2 (4.4–12.4) (n = 3)	3/3

\* When n = 3, the range is given in parentheses. ND, not done; Tg, transgenic.



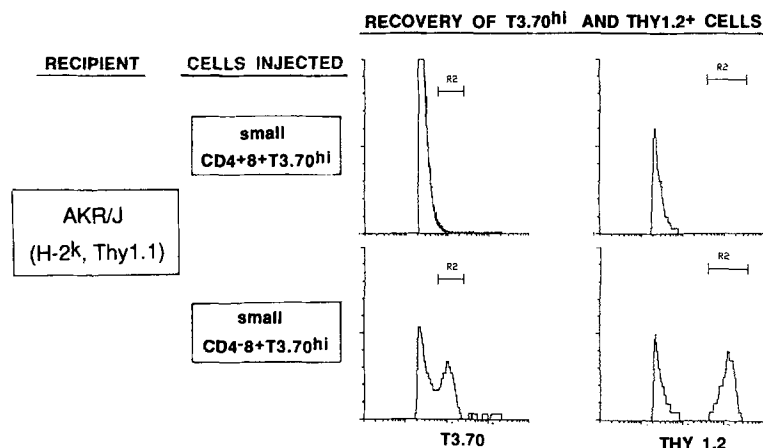
**Figure 3.** TCR transgenic CD4<sup>-</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> thymocytes can be recovered from H-2D<sup>b</sup>-deficient recipients. Small CD4<sup>-</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> thymocytes ( $3 \times 10^5$ , purity 99.5%) from H-2<sup>b</sup> TCR transgenic female mice were injected intrathymically into the indicated female recipients. 4 d later, the recipient thymuses were analyzed for the presence of T3.70<sup>hi</sup> cells as described in Materials and Methods. For percent recovery of T3.70<sup>hi</sup> cells, see Table 1. Broken lines mark the expression level (mean fluorescence) of TCR on cells recovered from the B6 recipient expressing positively selecting ligand for T3.70<sup>+</sup> TCR. Note the reduced expression level of T3.70<sup>+</sup> TCR on cells recovered from  $\beta 2m^{-/-}$  and BALB/c recipients that lack ligand for T3.70<sup>+</sup> TCR.

periments using CD4<sup>+</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> thymocytes from the TCR and bcl-2 double transgenic mice in which apoptosis of thymocytes is strongly suppressed (reference 13, and our own unpublished results). As shown in Fig. 5, differentiation of the double transgenic CD4<sup>+</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> thymocytes in  $\beta 2m^{-/-}$  and in BALB/c recipients is strongly inhibited as compared with differentiation occurring in B6 recipients. Although a substantial proportion of T3.70<sup>hi</sup> cells



**Figure 5.** Inhibition of differentiation of H-2<sup>b</sup> bcl-2/TCR bitransgenic CD4<sup>+</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> thymocytes in H-2D<sup>b</sup>-deficient recipients. Small CD4<sup>+</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> thymocytes ( $3 \times 10^5$ , purity 99.4%) from H-2<sup>b</sup> bcl-2/TCR bitransgenic female mice were injected intrathymically into the indicated female recipients. 4 d later, the recipient thymuses were analyzed for the presence of T3.70<sup>hi</sup> cells, and their CD4/CD8 phenotypes and level of HSA expression were determined as described in Materials and Methods. Broken vertical lines mark the highest expression level (mean fluorescence) of TCR and HSA molecules. Note that cells recovered from recipients lacking ligand for T3.70<sup>+</sup> TCR express reduced levels of TCR and high levels of HSA. For comparison, the expression level of HSA in the total population of recipient thymocytes is indicated by a dotted line. Numbers in quadrants indicate percentages. For percent recovery of T3.70<sup>hi</sup> cells, see Table 1.

recovered from  $\beta 2m^{-/-}$  and BALB/c recipients down-modulated CD4 coreceptor, they did not (in contrast to cells recovered from B6 recipients) down-modulate HSA molecules. These results strongly suggested that not only suppression of apoptosis but also end-stage differentiation of class I MHC-restricted CD8<sup>+</sup> T cells is dependent on continual engagement of the TCR, which cannot be substituted by engagement of coreceptors alone. However, the fact that some of the double transgenic CD4<sup>+</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> thymocytes



**Figure 4.** Small CD4<sup>+</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> thymocytes in contrast to small CD4<sup>-</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> thymocytes do not survive in the absence of continual engagement of the TCR by positively selecting ligand. Small CD4<sup>+</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> and small CD4<sup>-</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> thymocytes from H-2<sup>b</sup>, Thy1.2 TCR transgenic female mice ( $5 \times 10^5$ , purity >99%) were injected intrathymically into AKR (H-2<sup>k</sup>, Thy1.1) recipients. 4 d later, the recipient thymuses were analyzed for the presence of T3.70<sup>hi</sup> as well as Thy1.2<sup>+</sup> cells as described in Materials and Methods. Note the absence of T3.70<sup>hi</sup> and Thy1.2<sup>+</sup> cells in recipients injected with small CD4<sup>+</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> cells. This experiment was repeated twice with the same results.

down-modulated CD4 coreceptor in recipients lacking positively selecting ligands could indicate that the rescue from PCD is sufficient to allow at least some differentiation of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes activated by the initial TCR-mediated interaction, which in the absence of further TCR engagement is simply slowed down. Alternatively, since the injected cells themselves expressed H-2D<sup>b</sup> molecules, the down-modulation of CD4 coreceptors by some CD4<sup>+</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> thymocytes in  $\beta 2m^{-/-}$  and BALB/c recipients could result from interactions between intrathymically transferred thymocytes. To test these alternatives and to exclude formally the possibility that MHC incompatibility between donors and recipients has influenced our results, we performed experiments in which differentiation of small H-2<sup>d</sup> bcl-2/TCR bitransgenic CD4<sup>+</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> thymocytes positively selected in thymuses of BDF1 (H-2<sup>b/d</sup>) mice was compared after intrathymic transfer into BALB/c and B6 recipients. The results shown in Fig. 6 indicate that the great majority (86–91%) of H-2<sup>d</sup> (i.e., H-2D<sup>b</sup>) bitransgenic T3.70<sup>hi</sup> thymocytes recovered from BALB/c (H-2<sup>d</sup>) recipients remained CD4<sup>+</sup>CD8<sup>+</sup>, whereas the majority (64–83%) of T3.70<sup>hi</sup> cells recovered from B6 thymus were CD4<sup>-</sup>CD8<sup>+</sup>. These results suggest that down-modulation of the CD4 coreceptor on H-2D<sup>b</sup> double transgenic CD4<sup>+</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> thymocytes injected into H-2<sup>b</sup> thymus is due largely to interactions among injected thymocytes. Such TCR-mediated interactions with class I MHC molecules that can occur between thymocytes could explain the results of our

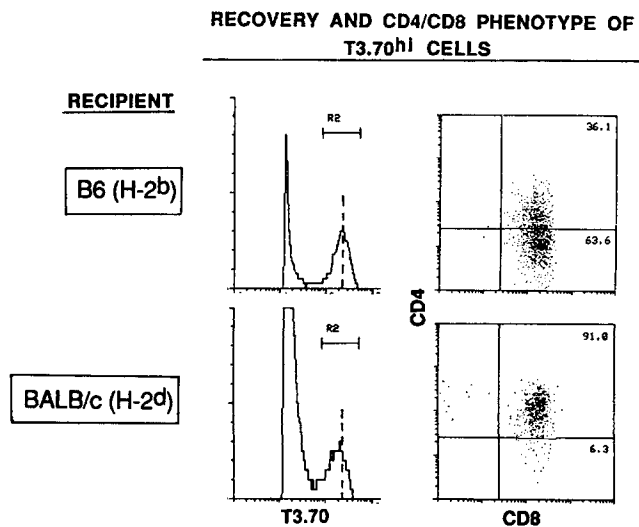
earlier *in vitro* experiments (27) as well as those of others (17), in which it was shown that CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>hi</sup> cells were able to complete their differentiation to CD4<sup>-</sup>CD8<sup>+</sup>T cells in culture in the apparent absence of thymic stromal elements. In the present experiments, somewhat less efficient differentiation of H-2<sup>d</sup> bitransgenic CD4<sup>+</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> thymocytes than of H-2<sup>b</sup> bitransgenic CD4<sup>+</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> thymocytes in B6 (H-2<sup>b</sup>) recipients (compare Figs. 5 and 6) may also reflect the fact that interactions among injected thymocytes contribute to the efficiency of differentiation. The small proportion of H-2<sup>d</sup> double transgenic CD4<sup>-</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> thymocytes that can be still detected in BALB/c recipients could at least in part represent contaminants of mature thymocytes originally present in purified population of CD4<sup>+</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> cells.

Thus, we conclude from these experiments that rescue from PCD and differentiation of mature class I MHC-restricted CD8<sup>+</sup>T cells from positively selected CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>hi</sup> thymocytes depend on continual interaction of the TCR with positively selecting ligand.

## Discussion

8 yr ago it was postulated that positive selection of T cells initiated by TCR-MHC interaction involves rescue from PCD (29). Although this notion received experimental support, the stage of development at which survival of positively selected cells becomes independent of TCR-MHC interactions remained undetermined. Moreover, the relationship between differentiation and suppression of apoptosis during positive selection as well as the role of TCR and/or CD4/CD8 coreceptors in transmitting signals responsible for these two effects has also remained unclear.

In the present study we have approached these questions by analyzing the fate of CD4<sup>+</sup>CD8<sup>+</sup> precursor thymocytes expressing class I MHC-restricted TCR after intrathymic injection into mice deficient in ligands for both the TCR and the CD8 coreceptor or for the TCR only. Using TCR transgenic or TCR/bcl-2 double transgenic mice as donors of positively selected CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>hi</sup> cells, we could examine the requirement for the continual engagement of the TCR versus the requirement for the continual engagement of coreceptors alone in rescuing from PCD and in promoting differentiation. We showed that within 2 d after transfer, CD4<sup>+</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> thymocytes of TCR transgenic mice vanished from the recipient thymus without generating any intrathymic progeny unless their TCR molecules were allowed to interact further with positively selecting H-2D<sup>b</sup> molecules. In the latter case, within 2–4 d the CD4<sup>+</sup>CD8<sup>+</sup>T3.70<sup>hi</sup>HSA<sup>hi</sup> thymocytes down-regulated expression of CD4 and HSA molecules and became the CD4<sup>-</sup>CD8<sup>+</sup>T3.70<sup>hi</sup>HSA<sup>lo</sup> thymocytes that were independent of further interactions with positively selecting ligands, at least for their short term (>4 d) survival. These results are compatible with conclusions drawn from DNA labeling studies (3) that the lifespan of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes is the same irrespective of whether or not they undergo positive selection: selected



**Figure 6.** Differentiation of CD4<sup>+</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> thymocytes rescued from PCD requires continual engagement of the TCR by positively selecting ligand. Small CD4<sup>+</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> thymocytes ( $3 \times 10^5$ , purity 98.5%) from bcl-2/TCR bitransgenic H-2<sup>d</sup> → H-2<sup>d/b</sup> female mice were injected intrathymically into the indicated female recipients. 4 d later, the recipient thymuses were analyzed for the presence of T3.70<sup>hi</sup> cells, and their CD4/CD8 phenotypes were determined as described in Materials and Methods. Broken lines mark the highest expression level (mean fluorescence) of TCR. Numbers in quadrants indicate percentages. In two other experiments the percentages of cells in the upper (CD4<sup>+</sup>CD8<sup>+</sup>) and the lower (CD4<sup>-</sup>CD8<sup>+</sup>) right quadrants recovered from B6 and BALB/c recipients were 14/83 versus 90/9 and 24/75 versus 86/13, respectively.

cells change their phenotype at the end of the 3.5-d period, whereas unselected cells die by apoptosis. Our present results complement these conclusions by indicating that the induction of differentiation by recognition of positively selecting ligand does not rescue CD4<sup>+</sup>CD8<sup>+</sup> thymocytes from PCD and that suppression of apoptosis is the end result of the positive selection, which requires continual interaction of the TCR with positively selecting ligand.

The requirement of CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>hi</sup> cells, positively selected by class I MHC-peptide complexes, for further interaction with these molecules cannot be fulfilled by providing the ligand for the CD8 coreceptor only. This conclusion is supported by the observation that in contrast to the allogeneic recipients expressing the ligand for T3.70<sup>+</sup> TCR (28), the allogeneic recipients lacking the ligand for T3.70<sup>+</sup> TCR but not for the CD8 coreceptor were unable to support development of intrathymically transferred, positively selected CD4<sup>+</sup>CD8<sup>+</sup>T3.70<sup>hi</sup> thymocytes. Thus, our present results provide the first direct evidence that positive selection of mature class I MHC-restricted CD8<sup>+</sup> T cells is dependent on continual engagement of the TCR by its ligand and that it cannot be substituted by interactions mediated by coreceptors alone not only during initiation (7, 13) but also at the subsequent stages of positive selection of these cells.

Our observation that mature CD8<sup>+</sup> thymocytes survive for at least 4 d in the thymic environment lacking positively selecting ligands but down-modulate the expression level of the TCR may suggest that mature T cells can also be dependent on interactions with self MHC molecules; not for their survival, but for maintaining the surface expression of the TCR at the appropriate level.

Whether the requirement for intrathymic expression of class I MHC molecules during maturation of the CD8<sup>+</sup> T cells expressing class II MHC-restricted TCR, reported by Kirberg et al. (18), reflects the need for CD8-class I MHC interaction not involving the TCR remains to be established. This possibility could be directly analyzed by an approach similar to that used in the current study, namely, by intrathymic transfer of the early postselection stages of thymocytes into recipients lacking the ligand for the TCR but not for CD8 molecules.

Our current experiments have not addressed the question of whether development of class I MHC-restricted CD8<sup>+</sup> T cells from CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>hi</sup> thymocytes requires coengagement of the TCR with CD8 coreceptor. The coreceptor function of CD8 molecules during positive selection of class I MHC-restricted CD8<sup>+</sup> T cells was demonstrated previ-

ously (30, 31), but in these studies, it was not determined whether coengagement of CD8 with the TCR by the same MHC molecule is required at all stages of positive selection. The requirement for involvement of CD4 and CD8 molecules during later stages of positive selection was documented in cases in which maturation of thymocytes that down-regulates the "wrong" coreceptor was induced by a transgene encoding the "correct" coreceptor (15, 21, 32), but the question of whether coengagement of the TCR was also required was not studied. The importance of coreceptors during initiation of positive selection was demonstrated in another recent study (22) which showed that not only maturation, but also generation of cells with "mismatched" CD4<sup>+</sup>CD8<sup>lo</sup> phenotype in class II MHC-deficient mice required the presence of CD8 molecules. However, none of the aforementioned studies allow one to conclude that further development of the positively selected CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>hi</sup> thymocytes is dependent on coreceptor function involving coengagement of the TCR. This question and that of the importance of coengagement of the TCR with CD8 coreceptor for survival and differentiation of developing thymocytes can be approached by transfer experiments similar to those reported here using recipients bearing mutation in the CD8-binding  $\alpha 3$  domain of class I MHC molecules; these were unavailable to us. Thus, the elucidation of the role of the coreceptors in promoting development of positively selected CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>hi</sup> thymocytes requires further study.

In the current study we have shown that sustained interaction of the TCR with its ligand is required not only for survival, but also for full maturation of developing T cells because, unlike in IL-3-dependent multipotent hematopoietic stem cell line (33), bcl-2-mediated suppression of apoptosis in unselected CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>lo</sup> (7, 13) and in positively selected CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>hi</sup> thymocytes does not lead to further differentiation in the absence of additional signals transmitted by the TCR.

In conclusion, our results indicate that positive selection cannot be regarded as a process that rescues immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes with potentially "useful" receptors from PCD and simultaneously or subsequently triggers or enables expression of intrinsically determined maturation program. Rather, it can be seen as a process that induces the differentiation program in potentially "useful" immature thymocytes by continuous TCR-mediated signaling and then rescues from PCD only those cells that successfully completed the whole differentiation program. The latter strategy of selecting useful T cells seems more prudent.

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