

## **Negative Selection of CD4<sup>+</sup>CD8<sup>+</sup> Thymocytes by T Cell Receptor-induced Apoptosis Requires a Costimulatory Signal that Can Be Provided by CD28**

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### **Summary**

CD4<sup>+</sup>CD8<sup>+</sup> thymocytes expressing self-reactive T cell antigen receptors (TCR) are deleted in the thymus as a consequence of TCR/self-antigen/major histocompatibility complex interactions. However, the signals that are necessary to initiate clonal deletion have not yet been clarified. Here we demonstrate that TCR engagement does not efficiently induce apoptosis of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes, although it generates signals that increase expression of CD5, a thymocyte differentiation marker. In fact, TCR signals fail to induce thymocyte apoptosis even when augmented by simultaneous engagement with CD4 or lymphocyte function 1-associated molecules. In marked contrast, signals generated by engagement of both TCR and the costimulatory molecule CD28 potently induce apoptosis of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes. Thus, the present results define a requirement for both TCR and costimulatory signals for thymocyte apoptosis and identify CD28 as one molecule that is capable of providing the necessary costimulus. These results provide a molecular basis for differences among cell types in their ability to mediate negative selection of developing thymocytes.

**T**olerance to self-proteins is maintained among T cells through the elimination or inactivation of clones which express antigen receptors reactive to self-antigen/MHC complexes (1, 2). Elimination of self-reactive immature T cells takes place in the thymus by clonal deletion which occurs via apoptosis (3–5). CD4<sup>+</sup>CD8<sup>+</sup> thymocytes, the major targets of clonal deletion, can be induced to undergo apoptosis both in vivo and in vitro through engagement of their antigen receptors by either intrathymic self-ligands or by antireceptor antibodies (6–13). However, it is not known whether induction of apoptosis requires signals in addition to those transduced by the TCR. In fact, clonal deletion of thymocytes is usually assayed in the presence of dedicated APCs that are capable of providing ligands for costimulatory molecules present on thymocytes. Whether APCs bearing costimulatory ligands are uniquely capable of mediating TCR-driven apoptosis of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes, or whether any cell type capable of presenting self-antigen/MHC complexes can mediate negative selection (14) is not clear.

In this report, we show that isolated TCR signals do not efficiently drive apoptosis of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes even when enhanced by coengagement with CD4 or LFA-1. However, TCR signals deliver a potent apoptotic stimulus when combined with signals provided by the costimulatory molecule, CD28. These results demonstrate that both TCR and

costimulatory signals are necessary to induce thymocyte apoptosis and indicate that only cells expressing costimulatory ligands can mediate negative selection.

### **Materials and Methods**

**Isolation of CD4<sup>+</sup>CD8<sup>+</sup> Thymocytes.** CD4<sup>+</sup>CD8<sup>+</sup> thymocytes were purified from young adult C57BL/6 thymuses by panning on anti-CD8 coated plates (15). More than 95% of the harvested cells were CD4<sup>+</sup>CD8<sup>+</sup>.

**Culture Conditions and Antibodies.** 24-well tissue culture plates were coated with antibody incubating them overnight at 4°C with 500  $\mu$ l of a 50  $\mu$ g/ml solution in PBS of mAb to TCR- $\beta$  (H57-597 [16]), mAb to CD3 $\epsilon$  (145-2C11 [17]), mAb to CD28 (37.51; Pharmingen, San Diego, CA [18]), mAb to CD4 (GK1.5 [19]), a 1:100 dilution in PBS of dialyzed ammonium sulfate precipitates of mAb to LFA-1 (M17/4 [20]) ascites or combinations thereof. Thymocytes ( $2.5 \times 10^6$ ) were cultured in 0.5 ml RPMI medium supplemented with  $5 \times 10^{-5}$  M 2-ME and 10% FCS that had been depleted of endogenous steroids by treatment for 30 min at 56°C with a final concentration of 0.5% Norit A charcoal and 0.05% dextran. Dexamethasone was added to a final concentration of  $10^{-6}$  where indicated. Where indicated (see Fig. 3), aliquots of cultured CD4<sup>+</sup>CD8<sup>+</sup> thymocytes were removed at 4 h, transferred to uncoated wells, and incubated for an additional 15 h in medium alone at 37°C.

**Staining and Flow Cytometry Analysis.** At the end of culture,

cells were analyzed by two color flow cytometry on a FACScan<sup>®</sup> using Consort 30 and Lysis II software (see Fig. 1) or on a FACScan<sup>®</sup> (all from Becton Dickinson & Co., Mountain View, CA) using institute software (see Figs. 2 and 3, and Table 1). Cells were stained in the first color with FITC-conjugated mAb to CD5 (Ly-1, Becton Dickinson & Co.) or, as a negative control, FITC-conjugated mAb to human CD3 $\epsilon$  (Leu-4, Becton Dickinson & Co.). For the second color, cells were exposed to 1  $\mu$ g/ml ethidium bromide (EtBr; Sigma Chemical Co., St. Louis, MO) for 30 min as described (21). Data are displayed as one color histograms. Dead cells were not electronically excluded during acquisition or analysis.

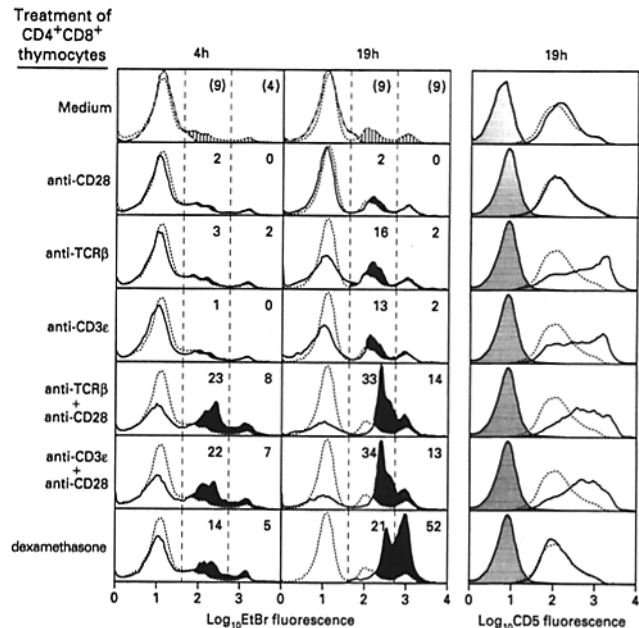
**DNA Fragmentation Assay.** CD4<sup>+</sup>CD8<sup>+</sup> thymocytes were cultured as indicated, harvested, stained with EtBr, and electronically sorted into EtBr<sup>-</sup> and EtBr<sup>int</sup> populations using a FACStar Plus<sup>®</sup> (Becton Dickinson & Co.). Genomic DNA was isolated (13) from sorted and unsorted thymocytes and from parallel groups of cells cultured with 10<sup>-6</sup> M dexamethasone. It was subsequently separated by electrophoresis through a 0.8% agarose gel containing 1  $\mu$ g/ml EtBr and visualized by UV fluorescence. Although EtBr can cause single stranded breaks in DNA when excited (22), it would not generate the ladder of fragments resulting from cleavage of internucleosomal double stranded DNA by endogenous endonucleases that is typical of apoptosis.

## Results

**Thymocyte Death Assay.** To assay thymocyte death *in vitro*, we stained thymocytes with EtBr as described (21, 23). EtBr stains nucleic acids, is rapidly taken up by thymocytes that are destined to die, and the fluorescence it emits can be measured on a single cell basis by flow cytometry. EtBr<sup>+</sup> thymocytes fluoresce with two different intensities (EtBr<sup>int</sup> and EtBr<sup>high</sup>), both of which represent dying cells (21). Indeed, these two populations are evident among CD4<sup>+</sup>CD8<sup>+</sup> thymocytes after *in vitro* treatment with dexamethasone, which is known to stimulate apoptosis of immature thymocytes (Fig. 1, *bottom*). EtBr<sup>int</sup> cells appeared as early as 4 h after treatment and EtBr<sup>high</sup> cells dominated the cultures after 19 h (Fig. 1, *bottom*). The frequency of nonviable cells as measured by trypan blue exclusion corresponded to the frequency of EtBr<sup>high</sup> cells (data not shown).

**TCR Signals Alone Do Not Efficiently Induce Apoptosis of CD4<sup>+</sup>CD8<sup>+</sup> Thymocytes.** To determine if TCR signals alone could induce thymocyte death, we isolated CD4<sup>+</sup>CD8<sup>+</sup> thymocytes and engaged their TCR with plate-bound anti-TCR- $\beta$  or anti-CD3 $\epsilon$  antibodies (Fig. 1, rows 3 and 4). The thymocytes responded to TCR cross-linking with an increase in surface expression of CD5 (Fig. 1, *right*), a marker of thymocyte maturation and activation (24, 25), indicating that intracellular signals were generated. However, TCR stimulation did not increase the proportion of EtBr<sup>+</sup> cells above that seen in control cultures after 4 h, and increased it only marginally after 19 h (Fig. 1, rows 3 and 4). Hence, signals generated by TCR/CD3 cross-linking alone do not efficiently induce death of susceptible CD4<sup>+</sup>CD8<sup>+</sup> thymocytes.

**CD28 Provides a Costimulatory Signal for TCR-mediated Apoptosis of CD4<sup>+</sup>CD8<sup>+</sup> Thymocytes.** The costimulatory molecule CD28 is expressed by most mature T cells and, when engaged, significantly enhances TCR-mediated prolifera-

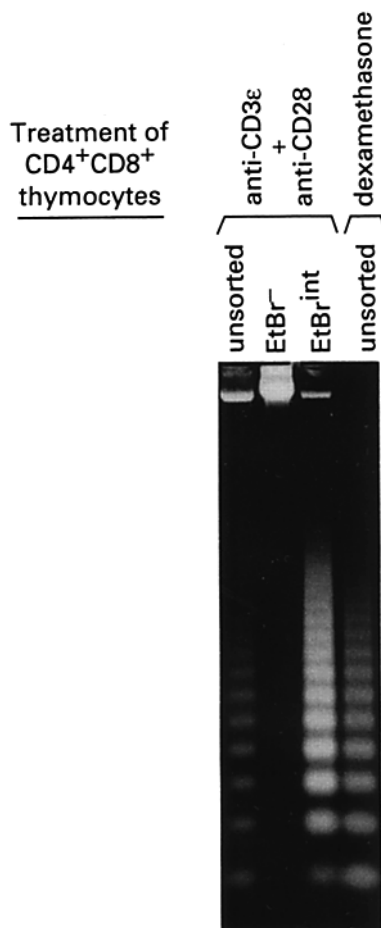


**Figure 1.** TCR-mediated apoptosis of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes requires costimulatory signals that can be provided by CD28. Purified CD4<sup>+</sup>CD8<sup>+</sup> thymocytes were cultured at 37°C for either 4 or 19 h in the presence of various plate-bound antibodies or dexamethasone. Total cell recovery was >95% and was unaffected by any of the treatments. Cells were stained with mAb to CD5 and EtBr and analyzed by flow cytometry. One-color histograms of EtBr staining revealed three populations of cells with different staining intensities: EtBr<sup>-</sup>, EtBr<sup>int</sup>, and EtBr<sup>high</sup>. In the first row, overlaid histograms represent staining profiles of cells cultured in medium alone at 4°C (*dashed line*) vs. cells cultured in medium alone at 37°C (*dotted line*). (*Hatched regions*) Differences in EtBr staining profiles. Numbers displayed in parentheses indicate percentages of EtBr<sup>int</sup> and EtBr<sup>high</sup> cells falling in these hatched regions and background death of unstimulated cells in 37°C cultures. In all other rows, panels display overlaid histograms representing staining profiles of treated cells (*solid lines*) vs. cells cultured in medium alone at 37°C (*dotted lines*). Differences in EtBr staining profiles between treated and control (medium alone) groups are indicated by dark shading and percentages of cells falling in these regions are displayed. Lightly shaded curves represent staining profiles of negative control antibody.

tion and effector activity among both CD4<sup>-</sup>CD8<sup>+</sup> and CD4<sup>+</sup>CD8<sup>-</sup> subpopulations (26–28). The involvement of CD28 costimulatory signals in the induction of apoptosis in immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes was suggested by the observations that (a) immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes express even higher levels of CD28 than mature T cells (17); and (b) APCs are potent inducers of thymocyte deletion and are now known to express the CD28 ligand, B7 (28–32). Consequently, we examined the ability of CD28 to act as a costimulatory molecule with the TCR/CD3 complex to induce the death of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes *in vitro* (Fig. 1). Engagement of CD28 alone had no effect (Fig. 1). However, engagement of CD28 together with either TCR- $\beta$  or CD3 $\epsilon$  induced an increase in the proportion of EtBr<sup>+</sup> thymocytes as early as 4 h after stimulation (Fig. 1, rows 5 and 6). To confirm that EtBr fluorescence identified apoptotic thymocytes, we examined genomic DNA from electron-

ically sorted EtBr<sup>-</sup> and EtBr<sup>int</sup> cells for fragmentation, a feature of apoptosis. Indeed, genomic DNA isolated from EtBr<sup>-</sup> cells was intact, whereas genomic DNA from EtBr<sup>int</sup> cells was highly fragmented (Fig. 2). Genomic DNA from EtBr<sup>high</sup> cells was also highly fragmented (data not shown).

Although the susceptibility of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes to death induced by TCR and CD28 costimulation was evident after 4 h of culture, it was most marked after 19 h (Fig. 1). To determine if TCR/CD28 signals had to be chronically applied to induce cell death, we exposed CD4<sup>+</sup>CD8<sup>+</sup> thymocytes to TCR and CD28 engagement for either 4 or 19 h and then examined their EtBr staining profiles after 19 h of culture (Fig. 3). The frequency of EtBr<sup>+</sup> cells was the same among thymocytes stimulated by TCR and CD28 coengagement for 4 h as it was among thymocytes stimulated for a full 19 h (Fig. 3, left). Hence, a 4-h stimulus was sufficient to commit susceptible thymocytes to die, although their commitment to undergo apoptosis was fully manifest only after



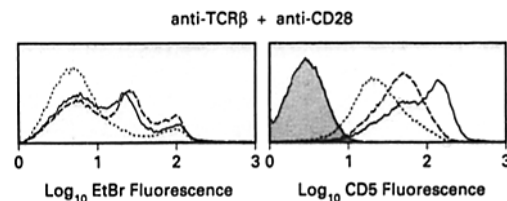
**Figure 2.** Assessment of genomic DNA in stimulated CD4<sup>+</sup>CD8<sup>+</sup> thymocytes. CD4<sup>+</sup>CD8<sup>+</sup> thymocytes were cultured with platebound anti-CD3ε and anti-CD28 for 14 h. Cells were then stained with EtBr and electronically sorted into EtBr<sup>-</sup> and EtBr<sup>int</sup> populations. DNA was extracted from these populations as well as from CD4<sup>+</sup>CD8<sup>+</sup> thymocytes treated in parallel with dexamethasone and electrophoresed on an agarose gel. The gel was stained with EtBr and visualized by UV light. Each lane represents DNA from 10<sup>6</sup> cells.

19 h of culture. In contrast, optimal CD5 upregulation required a continual TCR signal, for thymocytes stimulated by TCR and CD28 for 4 h expressed significantly lower levels of CD5 than those stimulated for 19 h (Fig. 3, right).

*Neither LFA-1 nor CD4 Provides a Costimulatory Signal for TCR-mediated Apoptosis.* Whereas engagement of CD28 provided costimulatory signals in CD4<sup>+</sup>CD8<sup>+</sup> thymocytes for TCR-induced apoptosis, this was not the case for other surface molecules expressed by CD4<sup>+</sup>CD8<sup>+</sup> thymocytes. Neither coengagement of CD4 nor LFA-1 with TCR-β increased the proportion of EtBr<sup>+</sup> cells, although both LFA-1 and CD4 synergized with TCR to increase CD5 expression significantly above that induced by TCR engagement alone (Table 1). These data demonstrate that both CD4 and LFA-1 can augment TCR signaling, but that neither provides a costimulatory signal for apoptosis of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes. Previous reports indicating that LFA-1 facilitates apoptosis of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes (33), together with our present results, suggest that LFA-1 acts indirectly by signaling APC to upregulate expression of ligands for bona fide costimulatory signaling molecules on CD4<sup>+</sup>CD8<sup>+</sup> thymocytes, such as CD28 (34).

## Discussion

This study reveals the inability of isolated TCR signals, even when augmented by coengagement with CD4 or LFA-1, to efficiently induce apoptosis of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes. Rather, induction of apoptosis requires both TCR signals and costimulatory signals that can be provided by CD28. These results provide a molecular basis for observations that dendritic cells, which constitutively express B7 (28, 31, 32), mediate clonal deletion much more efficiently than other cell types, including thymic epithelium (1, 29, 30). Our findings appear to conflict with recent observations that negative selection of thymocytes occurs even under conditions in which CD28 engagement is prevented (35–37). We suspect, however, that other as yet undefined surface molecules on CD4<sup>+</sup>CD8<sup>+</sup> thymocytes are also capable of providing costimulatory apoptotic signals, and that the costimulatory



**Figure 3.** CD4<sup>+</sup>CD8<sup>+</sup> thymocytes are committed to die within 4 h of receipt of apoptotic stimulus. CD4<sup>+</sup>CD8<sup>+</sup> thymocytes were cultured in the presence of platebound mAbs to TCR-β and CD28 for either 4 h (dashed lines) or 19 h (solid lines). Cells cultured with mAbs for 4 h were replated in the absence of antibody for an additional 15 h. All groups were harvested after a total of 19 h in culture and stained with EtBr or mAb to CD5. (Dotted lines) Staining profiles of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes cultured in medium alone are indicated by dotted lines. (Lightly shaded curve) Staining profile of negative control antibody.

**Table 1.** Neither CD4 nor LFA-1 Provides a Costimulatory Signal for Induction of TCR-mediated Apoptosis of CD4<sup>+</sup>CD8<sup>+</sup> Thymocytes

	EtBr <sup>+</sup> thymocytes	CD5 fluorescence
	%	$\Delta FU \times 10^{-3}$
Exp. 1		
Medium	(19)*	(1,266) <sup>‡</sup>
Anti-CD28	0	<0
Anti-CD4 <sup>+</sup>	1	<0
Anti-TCR- $\beta$	8	1,546
Anti-TCR- $\beta$ + anti-CD28	42	3,212
Anti-TCR- $\beta$ + anti-CD4	11	3,808
Exp. 2		
Medium	(20)	(634)
Anti-CD28	1	113
Anti-LFA-1	2	0
Anti-TCR- $\beta$	13	1,575
Anti-TCR- $\beta$ + anti-CD28	27	2,047
Anti-TCR- $\beta$ + anti-LFA-1	4	2,417

CD4<sup>+</sup>CD8<sup>+</sup> thymocytes were cultured for 18 h at 37°C with plate-bound antibodies as indicated. Cells were harvested and stained with EtBr or mAb to CD5 and analyzed by flow cytometry as described in Materials and Methods.

\* Percent EtBr<sup>+</sup> cells = percent EtBr<sup>int</sup> plus percent EtBr<sup>high</sup>. Percent EtBr<sup>+</sup> cells from control cultures in the absence of antibody is shown in parentheses. Numbers without parentheses represent the change in frequency of EtBr<sup>+</sup> cell induced by antibody treatment relative to control cultures.

<sup>‡</sup> Fluorescence intensity was quantitated in linear fluorescence units (FU). The numbers in parentheses represent CD5 fluorescence intensity of control cells cultured without antibody; numbers without parentheses represent the change in CD5 fluorescence induced by antibody treatment relative to control cells cultured without antibody. FU = cell frequency  $\times$  median intensity; median intensity was derived by conversion of median logarithmic channel numbers to linear units using a calibration curve empirically derived for each logarithmic amplifier used.

function of these other molecules is manifest when CD28 is not itself engaged. In fact, the low frequency of dying thymocytes that we observed after TCR stimulation alone may be attributable to suboptimal costimulation resulting from interthymocyte interactions.

The present observation that TCR signaling by itself does not induce cell death has important implications for our understanding of TCR-mediated selection events in the thymus. That TCR signaling has different consequences in CD4<sup>+</sup>CD8<sup>+</sup> thymocytes, depending upon the presence or

absence of costimulatory signals, suggests a basis for the difference between positive and negative selection processes. Positive selection may be initiated when the TCR is engaged in the absence of costimulation, whereas negative selection may occur as a result of a high-affinity TCR interaction in the presence of a costimulatory signal. In fact, TCR signaling in the absence of costimulation appears to have consequences normally attributed to positive selection such as increased CD5 expression as seen here and increased TCR assembly (unpublished results).

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