Immature Thymocytes Become Sensitive to Calcium-mediated Apoptosis with the Onset of CD8, CD4, and the T Cell Receptor Expression: A Role for *bcl-2*?

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Summary

During intrathymic negative selection by clonal deletion, crosslinking of the T cell receptor (TCR) induces cell death by delivering an apoptotic signal(s) to the nucleus along a calcium-dependent pathway. We investigated the reactivity of early precursor-containing thymocytes to Ca^{2+}-induced signals, and discovered a breakpoint in their sensitivity to calcium-mediated cell death (CMCD). $CD25^+CD8^-4^-$ TCR^- (triple negative [TN]) thymocytes stimulated with a calcium ionophore maintain their viability and precursor activity. By contrast, their immediate progeny, $CD25^-CD8^{lo}4^{lo}TCR\alpha\beta^{lo}$ (triple low [TL]) cells react to calcium elevation by abrogation of precursor activity and apoptotic cell death. This developmental difference is specific for CMCD, since both $CD25^+$TN and $CD25^-$TL cells are susceptible to steroid-induced apoptosis. The presence of *bcl-2* mRNA correlates directly to the resistance to CMCD—$CD25^+$TN cells express it and $CD25^-$TL cells do not. These experiments show that thymocytes become sensitive to Ca^{2+}-induced apoptosis as soon as they begin to express molecules that mediate thymic selection, and suggest that a concomitant downregulation of *bcl-2* may mediate this phenomenon.

TCR-generated, calcium-mediated signals are instrumental in inducing antigen-dependent proliferation of mature T cells (1) and in mediating intrathymic clonal deletion of immature, self-reactive thymocytes (2–4). This latter phenomenon, here termed calcium-mediated cell death (CMCD)[1], is a mainstay of the deletional T cell tolerance to intrathymically expressed self-antigens and is mediated by the induction of apoptosis (2–4). Apoptotic death process is characterized by membrane blebbing, nuclear condensation, and fragmentation of DNA into oligonucleosomal units [5], and is encountered in most tissues at some stage of their development (for a review see reference 6). Apoptosis can also be induced in thymocytes by a variety of physical and chemical stimuli (for a review see reference 7), the best studied of which is the effect of corticosteroids (5–7). The apoptotic signaling process induced by steroids is mediated via their nuclear receptor (8), and is most likely distinct from CMCD. In both cases, the ultimate effector of apoptotic DNA fragmentation is believed to be a Ca^{2+}-dependent endogenous endonuclease (5, 7).

T cell precursors that lack CD8 and the TCR and express little CD4 develop in the thymus via an ordered sequence of phenotypic changes into mature, TCR^{hi} cells of $CD8^+4^-$ or $CD8^-4^+$ phenotype (for a review see reference 9). Most thymocytes die at the $TCR^{int/lo}$ $CD8^{hi}4^{hi}$ double-positive (DP) stage, due to a lack of positive selection or negative selection by clonal deletion. Both processes are determined by the specificity of the TCR. Virtually nothing is known about the regulation of CMCD signaling during T cell development and its relationship to the onset of TCR-α/β receptor expression. Two main possibilities can be envisioned. First, the CMCD pathway may exist and be ready to react to Ca^{2+} elevation irrespective of the developmental stage of thymocytes. A prediction of this hypothesis would be that all thymocytes should be sensitive to CMCD. Second, the pathway does not exist, or cannot transform Ca^{2+} signals into apoptotic death until the onset of TCR expression. The implication of this hypothesis is that TCR^- and TCR^+ thymocyte subsets should differ in susceptibility to Ca^{2+}-induced apoptosis. Both types of subsets should, by contrast, be sensitive to apoptosis induced by TCR/Ca^{2+}-independent signals. We present findings consistent with the second hypothesis, and show evidence suggesting a role for *bcl-2* in mediating resistance to CMCD.

[1] *Abbreviations used in this paper:* CI, conversion index; CMCD, calcium-mediated cell death; CsA, Cyclosporin A; Dex, dexamethasone; DN, double negative; DP, $CD8^+4^+$ double positive; FCM, flow cytometry; GARIG, goat anti-rat IgG; HSA, heat stable antigen; PI, propidium iodide; SA, streptavidin; TN, $CD8^-4^-TCR^-$ triple negative; TL, $CD8^{lo}4^{lo}TCR\alpha\beta^{lo}$ triple low.

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Materials and Methods

Mice. Female BALB/c, C57BL/6 (The Jackson Laboratory, Bar Harbor, ME), B6.PL-thy-1^a Cy, B6-Ly-5.2 (Memorial Sloan-Kettering animal facility), and B6D2 F_1 mice (National Cancer Institute animal facility, Frederick, MD) were used at 4–8 wk of age.

Reagents. The original source, purification, and conjugation to fluorochromes of rat mAbs 3.168, αCD8; 2B6, αCD4; PC61, αCD25; IM 7.1, αCD44; J11d.2 α heat stable antigen (αHSA); and M1/69, αHSA, are referenced in (10–12). Allele-specific IgG2a antibodies against Ly-5.1 (104-2.1) and Ly-5.2 (A20-1.7) (13), obtained from Drs. S. Kimura and U. Hammerling (Memorial Sloan-Kettering Cancer Center), were detected using goat anti–mouse IgG2a-FITC antibody (Fisher Scientific, Malvern, PA). Purified goat anti–rat IgG (GARIG, Sigma Chemical Co., St. Louis, MO), αCD4-PE, and αThy-1.2-FITC (Becton Dickinson & Co., Mountain View, CA), αCD8-B (Coulter Corp., Hialeah, FL) streptavidin-TriColor (SA-TC; Caltag Laboratories, San Francisco, CA), PMA (Sigma Chemical Co.), ionomycin (Boehringer Mannheim Corp., Indianapolis, IN) and a guinea pig serum, a source of complement (GIBCO BRL, Gaithersburg, MD), were purchased from commercial sources. Cyclosporin A (CsA) and dexamethasone (Dex) were obtained from the pharmacy of the Memorial Sloan-Kettering Cancer Center.

Isolation and Stimulation of Thymocyte Subsets. HSA$^+$ double negative (DN) thymocytes were purified from total thymocytes by a combination of mAb plus C′ depletion and positive selection by panning, as described (10–12). HSA$^+$DN cells were then panned on GARIG-coated dishes. GARIG-adherent fraction contained the cells that survived mAb and complement treatment but were coated with the rat αCD8 and αCD4 antibodies, owing to low expression of CD4 and CD8. These CD8^{lo}4^{lo}TCR^{lo} (triple low [TL]) cells were used for further studies. GARIG-nonadherent cells were further positively selected on PC61-coated plates. Adherent cells were recovered and used as CD25$^+$ CD8$^-$4$^-$TCR$^-$ (triple negative [TN]) cells in experiments. Cross-contamination among the two subsets was negligible (CD25$^+$TN cells were usually >95% CD25^{hi}, whereas CD25$^-$TL cells contained 4–12% CD25^{lo} cells) as reported previously [11, 12]). Alternatively, J11d-adherent cells were sorted on the basis of CD25 versus CD44 expression into CD25$^+$CD44$^-$ (TN) and CD25$^-$CD44$^-$ (TL) fractions (14, 15). To isolate mature thymocytes, cells were treated with two cycles of J11d plus C′. These cells were routinely <1% HSA$^+$.

In vitro culture of thymocyte subsets was described previously (10). Concentration of stimuli was: ionomycin, 400 ng/ml; PMA, 10 ng/ml; CsA, 1 μg/ml; and dexamethasone, 10^{-7} M. These concentrations were determined not to be nonspecifically toxic to cells (data not shown). To maximize the action of CsA, cells were pretreated with CsA 1 h before addition of ionomycin. CsA was continuously present in culture. After the culture, cells were washed, counted, and used in the assays described below.

In Vitro and In Vivo Precursor Assays. These assays were described before (10–12). For the in vitro assay, cell phenotypes were analyzed by flow cytometry (FCM) at endpoint. Conversion index (CI) was determined by dividing the percentage of DP with the percentage of DN cells. This was empirically found to be the most accurate measure of conversion that can easily be used to compare different experiments. For the in vivo assay, thymocytes of host mice (congenic at Thy-1 or Ly-5 loci) were analyzed at indicated times after intrathymic injection of donor-type thymocyte subsets for the presence of donor-derived cells using Thy-1 or Ly-5 allele-specific mAbs.

DNA Fragmentation Assay. A quantitative assay of the DNA content in stimulated thymocyte subsets (16) and the isolation of nuclei (17) were described before. Nuclei equivalent to 1.5 × 10^6 cells were incubated with or without 10 mM $MgCl_2$ and 5 mM $CaCl_2$. After the incubation, propidium iodide (PI) was added at 20 μg/ml. P_1 fluorescence was measured using a FACScan® flow cytometer (Becton Dickinson & Co.). The percentage of cells undergoing DNA fragmentation was determined as the number of cells with DNA content in the subdiploid range characteristic of apoptotic cells.

FCM Analysis and Sorting. Two-color FCM analysis was performed on fresh and cultured thymocytes and their subsets. 5–50 × 10^4 cells were simultaneously stained with directly conjugated or biotinylated mAbs and the latter revealed by conjugated SA. Dead cells and debris were excluded by selective forward and 90° scatter gating or PI (1 μg/ml) with essentially the same results. 2–10 × 10^3 cells were scored per sample, using a FACScan® instrument. Markers were set to denote fluorescence above that of the background obtained with no antibody or with isotype-matched controls (Fisher Scientific). LYSYS II software (Becton Dickinson & Co.) was used for data analysis. Sorting was performed on HSA$^+$ DN cells stained with αCD44- and αCD25-specific mAb to isolate CD44$^-$CD25$^-$ (CD25$^-$TL) and CD44$^-$CD25$^+$ (CD25$^+$TN) cells. Purity was >99% upon reanalysis.

RT-PCR of bcl-2 mRNA. This assay was performed exactly as previously described (18). The reactions underwent 45 cycles of PCR amplification (denaturation at 94°C for 1 min; primer annealing at 61°C for *bcl-2* and at 55°C for β-actin, for 2 min; primer extension at 72°C for 3 min; and final extension at 72° for 7 min). The forward and reverse primers for *bcl-2* were as in reference 19; for β-actin, F(5′-GTG GGG CGC CCC AGG CAC CA-3′) and R(5′-CTC CTT ATT GTC ACG CAC GAT TTC-3′). All were used at a final concentration of 0.2 μM.

Results

Differential Response of Immature Thymocyte Subsets to Ca^{2+} Elevation. We isolated CD25$^-$ CD8$^-$4$^-$TCR$^-$ (CD25$^+$ TN) thymocytes and their immediate progenitors, CD25$^-$ CD8^{lo}4^{lo}TCRαβ^{lo} triple low (CD25$^-$TL) cells and stimulated them overnight with a calcium ionophore ionomycin. The viability of untreated (control) cells varied between 30 and 90% of the input and was usually slightly higher in CD25$^+$TN than in CD25$^-$TL cells (67.1 ± 16.4, n = 8, and 51.5 ± 20.4, n = 14, respectively), but the differences were never greater than 20% within the same experiment. CD25$^+$TN thymocytes did not change viability whether cultured without stimuli, or in the presence of PMA or ionomycin (Table 1 and data not shown). The number of cells recovered from the CD25$^-$TL cultures remained constant relative to control in the presence of PMA. By contrast, ionomycin dramatically reduced the viability of CD25$^-$TL subset (Table 1).

Phenotypic conversion of these two subsets into CD8^{hi}4^{hi} (DP) cells is shown in Table 1. CD25$^+$TN cells cannot undergo phenotypic change in vitro (10), and none of the stimuli used in this study, alone or in combination, could induce them to do so. A distinguishing feature of CD25$^-$TL thymocytes is their ability to convert to DP cells in vitro (10, 11), and both PMA, ionomycin, and the combination of the two completely abrogated this conversion (Table 1, Fig. 1,

Table 1. *In Vitro Survival and Conversion and In Vivo Precursor Activity of Early Thymocyte Subsets Stimulated with PMA and Ionomycin (Iono)*

In vitro	Subset	Stimulus	Conversion index	Survival (Percent control)	In vivo	Subset transferred	Stimulation	Percent donor-derived cells/thymus	No. of donor-derived cells/thymus ($\times 10^6$)
Exp. 1	$CD25^-TL$	–	0.89	100	Exp. 1	$CD25^-TL$	–	4.6; 3.4; 8.0	1.3; 0.9; 2.4
		PMA	0.10	104.2			Iono	0.4; 0.2	0.1; 0.1
		Iono	0.10	6.8					
	$CD25^+TN$	–	0.01	100	Exp. 2*	$CD25^-TL$	–	43.1	11.5
		PMA	0.01	101.6			Iono	0.2	0.2
		Iono	0.01	98.3		$CD25^+TN$	–	69.8	24.1
							Iono	64.7	26.1
Exp. 2	$CD25^-TL$	–	2.35	100	Exp. 3	$CD25^+TN$	–	36.2; 38.0; 0.9	4.0; 9.5; 0.4
		Iono	0.16	16.3			Iono	10.6; 5.1; 13.9; 14.4; 26.7	2.5; 1.3; 4.6; 3.6; 9.1
	$CD25^+TN$	–	0.01	100					
		Iono	0.01	104.7		No transfer	–	0.3	0.1

Thymocyte subsets were treated and analyzed as described in Materials and Methods and Fig. 1. Viability was determined by trypan blue exclusion, and ranged between 43 and 75% between experiments for untreated controls. Control viability was taken as a reference point of 100%. For the in vivo studies, 2–7 × 10^5 cells injected into thymuses of recipient mice (two to five/group, B6.PL in Exp. 1 and 2 and B6-Ly5.2 in Exp. 3). 9–13 d later, individual host thymuses were analyzed for the presence of donor-derived progeny by FCM, and the results expressed as percent donor-derived cells, and as absolute numbers of donor-derived cells, calculated from the total number of thymocytes and the percent donor-derived cells. * Pooled thymocytes (four/group) were analyzed. In these experiments, $CD25^+TN$ population was 88–99% $CD25^{hi}$ and CD25 TL cells contained between 1 and 10% $CD25^{lo}$ cells.

and data not shown). PMA was used as a positive control expected to inhibit conversion but not to induce apoptosis, since it was recently shown that PMA inhibits conversion by inducing degradation of CD4 and CD8 mRNA (20). The doses of PMA and ionomycin that affect $CD25^-TL$ cells correspond to the ones required to elicit a proliferative response in mature T cells (data not shown). Time course experiments demonstrated that each reagent needed at least 3–6 h to inhibit conversion. Together, they acted synergistically and caused a profound inhibition of conversion (21, and data not shown). Finally, the addition of calcium chelators completely abrogated the effects of ionomycin, showing that the abrogation of conversion and cell death are due to Ca^{2+} influx and not to nonspecific cytotoxicity of ionomycin (data not shown).

To test the effect of calcium elevation on the in vivo precursor capacity of the two early subsets, control and stimulated cells were introduced into the thymuses of Thy-1 or Ly-5 congenic, irradiated recipients. Cultured cells from both subsets retain detectable, although diminished, in vivo precursor activity compared to freshly isolated cells (14, Table 1, and data not shown). To avoid bias due to variation in the efficiency of intrathymic injection, we performed individual analyses of injected thymuses. For both subsets, the results of intrathymic assays strictly paralleled the conversion and viability data. Namely, ionomycin completely abrogated the in vivo

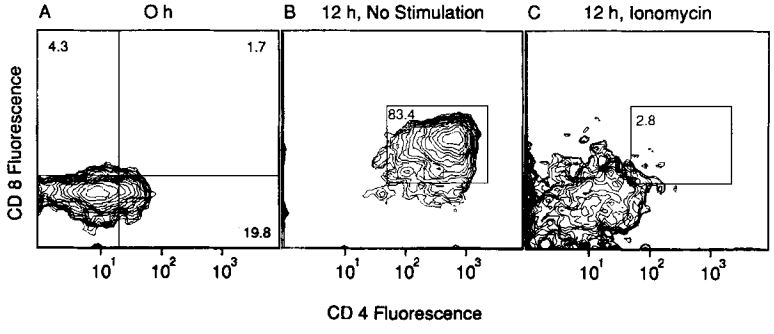

Figure 1. Ionomycin inhibits in vitro conversion of $CD25^-TL$ thymocytes into DP cells. Purified $CD25^-TL$ thymocytes were stained immediately after isolation (*A*), and cultured without stimulation (*B*) or in the presence of ionomycin (*C*). Expression of CD8 and CD4 was determined by FCM. Data from 5 × 10^3 cells are shown as 80% logarithmic contour plots (threshold = 3). (*A*) Markers are set to denote fluorescence above that of control, and it can be seen that TL cells express low levels of CD8 and CD4. (*B* and *C*) Region is drawn to denote bright DP cells. The results are representative of more than 20 experiments.

Table 2. *DNA Fragmentation in Immature Thymocyte Subsets and Their Nuclei in Response to Calcium Elevation*

Thymocyte subsets			Nuclei		
Subset	Treatment	% DNA fragmentation*	Nuclei from	Ion added	% DNA fragmentation*
$CD25^-TL$	–	48.5	$CD25^-TL$	Nil	19.8
	Iono	71.3		Mg^{2+}	8.2
	Dex	72.8		Ca^{2+}	88.1
	CsA	48.8		Ca^{2+} + Mg^{2+}	ND
	CsA + Iono	70.9			
$CD25^+TN$	–	35.2	$CD25^+TN$	Nil	35.3
	Iono	24.2		Mg^{2+}	8.4
	Dex	81.6		Ca^{2+}	85.4
	CsA	ND		Ca^{2+} + Mg^{2+}	87.5
	CsA + Iono	ND			

Early thymocyte subsets were cultured with indicated stimuli in vitro, and their DNA content assayed as described in Materials and Methods. $CD25^+$ TN thymocytes were 89.7% $CD25^{hi}$, and $CD25^-TL$ cells contained 11.9% $CD25^{lo}$ cells. CI for TL cells was: control, 17.1; iono, 0.4; Dex, ND; CsA, 16.5; iono + CsA, 0.1; and for all TN < 0.1. These cells were also used for the experiment shown in Fig. 2. Results are representative of four experiments. Nuclei were isolated, treated, and tested as described in Materials and Methods. CI was 2.8 for TL and 0.03 for TN cells. At the beginning of culture neither subset contained any DP or SP cells. They were >99% HSA^+, and TL and TN cells were 5.7 and 97.6% positive for CD25, respectively.

* Determined as percent cells with clearly subdiploid DNA content, as described (20).

precursor activity of the $CD25^-TL$ cells. By contrast, this agent caused no changes in the precursor activity of $CD25^+TN$ cells (Table 1).

Elevation of Intracellular Ca^{2+} Induces Apoptosis in $CD25^-$ TL but not in $CD25^+TN$ Cells. Next, we investigated the mechanism of cell death in thymocyte subsets after stimulation using a quantitative flow cytometric assay of DNA ploidy (16). Both cell subsets exhibited high background DNA fragmentation (30–55% in six experiments), consistent with spontaneous death of these cells outside the thymus (Table 2). PMA, used as a control reagent, did not affect fragmentation of either subset (data not shown). $CD25^-TN$ cells markedly increased DNA fragmentation in the presence of ionomycin. By contrast, ionomycin could not induce fragmentation in $CD25^+TN$ cells. Dexamethasone treatment induced a near-complete DNA fragmentation in both subsets, demonstrating that $CD25^+TN$ cells are resistant to CMCD but are not generally resistant to apoptosis. Together, these results indicate that a specific developmental difference in sensitivity to CMCD exists between $CD25^+TN$ and $CD25^-TL$ cells. Importantly, CsA could not reverse the Ca^{2+}-induced apoptosis of TL cells suggesting that calcineurin (22, 23) is not involved in the transduction of Ca^{2+}-mediated apoptotic signals.

Several control experiments were performed to ascertain that FCM measurements detect apoptosis, rather than nonspecific cell death. Microscopic appearance of Ca^{2+}-stimulated $CD25^-TL$ and steroid-treated $CD25^+TN$ and $CD25^-TL$ cells, but not of Ca^{2+}-stimulated $CD25^+TN$ thymocytes, was consistent with apoptosis. DNA laddering was always detected in apoptotic samples and never in freshly isolated cells, but was quantitatively noninformative owing to the high background in cultured cells. Finally, the process was inhibited by cyclohexamide in all samples and by EGTA in calcium-stimulated ones (data not shown).

A Ca^{2+}-dependent endogenous endonuclease was implicated as the effector of fragmentation (5, 7). To test whether the effector(s) of the apoptotic pathway exists and is functional in $CD25^+TN$ cells, we isolated nuclei from $CD25^-$ TL and $CD25^+TN$ thymocytes, and incubated them in the presence or absence of Ca^{2+} or Mg^{2+}. Control magnesium ions did not induce fragmentation above that of the background (Table 2). By contrast, calcium ions induced a profound fragmentation of DNA in nuclei isolated from both subsets (Table 2). The resistance of $CD25^+TN$ cells to CMCD could in theory be due to the possibility that these cells poorly elevate Ca^{2+} in response to ionomycin. The analysis of Ca^{2+} fluxes in Fluo-3–loaded thymocyte subsets revealed, however, that both subsets are able to mobilize equally high Ca^{2+} levels (data not shown). There is, therefore, no difference between the two subsets at the most proximal and most distal steps of the CMCD pathway.

Expression of bcl-2 in Early Thymocyte Subsets Directly Correlates to Their Resistance to CMCD. Protooncogene *bcl-2* was recently shown to play a role in T cell apoptosis (24, 25). We therefore examined the presence of *bcl-2* mRNA in thymocyte subsets by sensitive RT-PCR analysis. The message was absent in total thymocytes (Fig. 2, lane *6*), strong in splenocytes (Fig. 2, lane *10*) and detectable, but at low level, in mature HSA^- thymocytes. Among early thymocyte subsets,

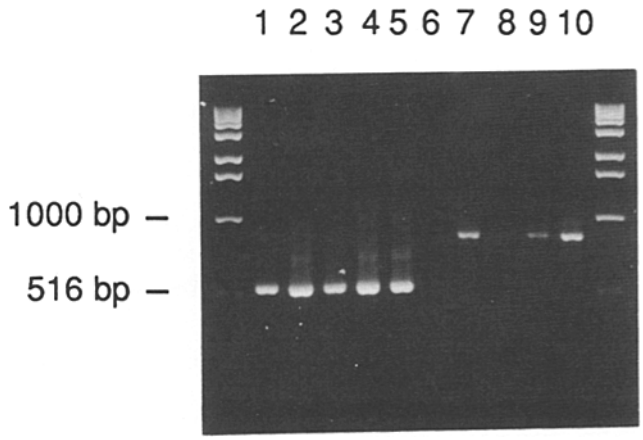

Figure 2. RT-PCR detection of *bcl-2* mRNA in thymocyte subsets. β-actin (lanes *1–5*) and *bcl-2* (lanes *6–10*) mRNA was detected by RT-PCR in total cellular RNA isolated from: unfractionated thymocytes (lanes *1* and *6*); CD25$^+$TN thymocytes (lanes *2* and *7*); CD25$^-$TL thymocytes (lanes *3* and *8*); HSA$^-$ thymocytes (lanes *4* and *9*); and total splenocytes (lanes *5* and *10*). Molecular weight markers (1-kb DNA ladder; GIBCO BRL) are shown on both sides in unmarked lanes, and the position of 1-kb and 516-bp markers denoted to the left. Primers were designed to amplify a 860-bp fragment of *bcl-2* and a 548-bp fragment of β-actin. Similar results were obtained in four other experiments. Purities and CI are shown in Table 2.

CD25$^+$TN cells expressed high levels of *bcl-2* mRNA (Fig. 2, lane 7). By contrast, we could not detect *bcl-2* mRNA in CD25$^-$TL thymocytes (Fig. 2, lane *8*). RNA quantities used in this experiment were comparable, based on the RT-PCR amplification of β-actin (Fig. 2, lanes *1–5*). These results suggest that the pattern of expression of *bcl-2* may account for the drastic difference in sensitivity of early thymocyte subsets to Ca^{2+}-induced signals. Moreover, the results of testing of other thymocyte subsets (Fig. 2) suggest that the expression of *bcl-2* may be restricted to the earliest, TCR$^-$, and the most mature, HSA$^-$ stages of intrathymic development. This is because no *bcl-2* message can be detected in total thymocytes, more than 80% of which are DP.

Discussion

It is generally believed that calcium is a critical second messenger in the induction of apoptosis via the TCR (2–4). Ca^{2+} signaling is therefore considered to form the biochemical basis of negative intrathymic selection, but the downstream signal transduction after the transmembrane influx of Ca^{2+} is poorly understood. Another type of poorly understood death by apoptosis that could be mediated by Ca^{2+} is even more frequent in the thymus and is due to a lack of positive selection (death by "neglect"). Here we show that CD25$^+$TN thymocytes display a selective resistance to Ca^{2+}-mediated apoptosis. As soon as the TCR and the accessory molecules are expressed on developing T cells, this resistance is lost, and the cells become exquisitely sensitive to the elevation of intracellular calcium, just as their immediate progeny, TCR$^{lo/int}$ DP thymocytes. CD25$^+$TN cells are not generally resistant to apoptosis, as steroids readily induce their death. Since steroids operate via a unique nuclear receptor(s) (8), steroid-induced apoptosis should be quite distinct from the induction of apoptosis via the TCR, during which the Ca^{2+}-generated signal presumably travels through the cytoplasm. In that respect, it is important to determine if and at what point do CMCD and steroid-induced apoptotic pathways intersect and/or converge.

We did not detect differences between CD25$^+$TN and CD25$^-$TL cells in the most proximal (Ca^{2+} mobilization) and the most distal (endonuclease activation) points of the apoptotic pathway. An obligatory player in the Ca^{2+}-mediated T cell activation, calcineurin, appears not to be required for the transduction of a Ca^{2+}-mediated apoptotic signal in CD25$^-$TL cells, judging by the inability of CsA to inhibit apoptosis. CsA was shown to abrogate apoptosis and negative selection in some (4, 26, 27) but not other (28, 29) experimental systems. Our results are consistent with the proposal of Ucker et al. (29) that CsA cannot inhibit apoptosis induced by a single signal. Ca^{2+}-mediated, calcineurin-independent induction of apoptosis may suggest the existence of independent and parallel pathways of Ca^{2+} action: one would lead to T cell activation, the other to thymocyte death.

What biochemical change(s) dictates the transition from CMCD resistance to sensitivity? An abrupt cessation of *bcl-2* mRNA expression in CD25$^-$TL cells correlates with the onset of sensitivity to apoptosis. The role of *bcl-2* in blocking apoptosis is well established (19, 24, 25). Our results document its expression in early (CD25$^+$TN) and late (HSA$^-$), but not intermediate (CD25$^-$TL and DP) thymocytes. *bcl-2* could therefore play a major role in the developmental regulation of CMCD. Since a deregulated overexpression of *bcl-2* does not appear to grossly perturb clonal deletion (24, 25), and in light of our results indicating a lack of or very low expression of this molecule in selectable (DP) thymocytes, *bcl-2* probably does not play a significant physiological role in negative selection. Furthermore, in our studies, *bcl-2* does not protect CD25$^+$TN cells from steroid-induced death. This is in contrast with the observation that in transgenic animals *bcl-2* prevents steroid-induced death of unseparated thymocytes (24, 25). The most likely explanation of this discrepancy is that levels of *bcl-2* expression were supraphysiological in thymocytes of transgenic animals, whereas our study did not manipulate physiological levels of *bcl-2* expression.

We propose that the physiological role of *bcl-2* may be to make early thymocytes and mature T cells relatively resistant to apoptosis, and that its absence renders intermediate thymocytes susceptible to death by neglect, unless they are rescued by positive selection. Whether death by neglect involves Ca^{2+} as the key messenger and what cell surface structures induce this process is under study. The role of bcl-2 and other molecules such as Fas (30) and c-*myc* (31) in T cell development and whether they act using the same or different signaling pathways are questions of paramount importance to our understanding of thymocyte apoptosis.

Resistance to TCR-mediated deletion was demonstrated previously in a subset of fetal DP thymocytes (32, 33), using differential susceptibility of these cells to anti-CD3 versus

anti-TCR-α/β mAb treatment. But these results have not been reproduced in the adult thymus to date. We now show that early adult thymocyte populations are protected from Ca^{2+}-mediated cell death until they begin to express CD8, CD4, and TCR. The expression of even very low amounts of these molecules correlates with an exquisite sensitivity to Ca^{2+} elevation. Calcium-driven cell death is therefore a "privilege" bestowed upon thymocytes that reach the selection stage, perhaps by downregulation of *bcl-2* expression.

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Note added in proof: While this manuscript was in press, two groups have assayed the expression of the Bcl-2 protein in human (Gratiot-Deans et al., *J. Immunol.* 151:83, 1993) and human and mouse (Veis et al., *J. Immunol.* 151:2546, 1993) thymocytes. Although some differences exist between these findings and the ones reported here (most likely owing to different assays used to detect Bcl-2) the common finding of all three studies is that Bcl-2 is expressed at high levels early and late in thymocyte development and at low levels in DP thymocytes.

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