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$^+$4$^+$TCR$^+$ (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.

T cell-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. 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.

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$^1$ 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$^+$4$^+$TCR$^+$ tripod low.
Materials and Methods

**Materials.** Female BALB/c, C57BL/6 (The Jackson Laboratory, Bar Harbor, ME), B6.PL-thy-1* Cy, B6-Ly-5.2 (Memorial Sloan-Kettering animal facility), and B6D2 F1 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 a 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-FITC, 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, as a source of complement (GIBCO BRL, Gaithersburg, MD), were purchased from commercial sources. Cyclosporin A (CsA) and dexamethasone (Dex), obtained from Drs. S. Kimura and U. Hammerling (Memorial Sloan-Kettering Cancer Center), were analyzed at indicated times after intrathymic injection of donor-type thymocyte subsets. 5–50 x 10^5 cells were simultaneously stained with directly conjugated mAbs and the latter revealed by conjugated SA.

**FACScan® Flow Cytometry.** FACScan® (Becton Dickinson & Co.) was used for data analysis. Sorting was performed on HSA+ DN cells stained with αCD4- and αCD25-specific mAb to isolate CD4−CD25− (CD25−TL) and CD4+CD25+ (CD25+TN) cells. Purity was >99% upon reanalysis.

**RTPCR 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°C for 7 min). The forward and reverse primers for bcl-2 were as in reference 19; for β-actin, F(5'-GTT GGG CGC CCC AGG CAC CA-3') and R(5'-CTT ATT ATT GTG AGG CAC CAG TTT-3'). All were used at a final concentration of 0.2 μM.

**Results.**

**Differential Response of Immature Thymocyte Subsets to Ca2+ Elevation.** We isolated CD25−CD8−4+TCR− (CD25+TN) thymocytes and their immediate progenitors, CD25−CD8−4+TCRαβ+ 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 CD8hiCD4hi (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). CD25− and CD25+TN thymocytes are their immediate progenitors, CD25−CD8−4+TCRαβ+ 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).

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| In vitro Subset | Stimulus | Conversion index (Percent control) | Survival | In vivo Subset transferred | Stimulation | Percent donor-derived cells/thymus | No. of donor-derived cells/thymus (× 10⁶) |
|----------------|----------|------------------------------------|----------|---------------------------|-------------|-----------------------------------|------------------------------------------|
| 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     |                           |             |                                   |                                         |
| 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; 2.5; 1.3; 4.6; 14.4; 26.7 | 3.6; 9.1                                |
| CD25⁺TN        | –        | 0.01                               | 100      |                           |             |                                   | 0.3                                     | 0.1                                     |
|                | Iono     | 0.01                               | 104.7    |                           | No transfer |                                   |                                         |

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 × 10⁶ 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⁻ cells.

Thymocytes are further analyzed in Table 1. The data show that the in vitro survival and conversion of CD25⁻TL thymocytes is not significantly affected by the presence of ionomycin (data not shown). The addition of calcium chelators completely abrogated the effects of ionomycin, showing that the abrogation of conversion and cell death are due to Ca²⁺ 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 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 × 10⁴ cells are shown at 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.

![Figure 1. Ionomycin inhibits in vitro conversion of CD25⁻TL thymocytes into DP cells.](image-url)
Table 2. DNA Fragmentation in Immature Thymocyte Subsets and Their Nuclei in Response to Calcium Elevation

| Subsets       | Treatment | % DNA fragmentation* | Nuclei from Ion added | % DNA fragmentation* |
|---------------|-----------|----------------------|-----------------------|----------------------|
| CD25⁻ TL     | -         | 48.5                 | CD25⁻ TL              | 19.8                 |
|               | Iono      | 71.3                 | Mg²⁺                  | 8.2                  |
|               | Dex       | 72.8                 | Ca²⁺                  | 88.1                 |
|               | CsA       | 48.8                 | Ca²⁺ + Mg²⁺           | ND                   |
|               | CsA + Iono| 70.9                 |                       |                      |
| CD25⁺ TN     | -         | 35.2                 | CD25⁺ TN              | 35.3                 |
|               | Iono      | 24.2                 | Mg²⁺                  | 8.4                  |
|               | Dex       | 81.6                 | Ca²⁺                  | 85.4                 |
|               | CsA       | ND                   | Ca²⁺ + Mg²⁺           | 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⁻ and CD25⁺ TL cells contained 11.9% CD25⁺ 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).

Elevation of Intracellular Ca²⁺ 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⁻ and CD25⁺ TL cells. Importantly, Ca²⁺ could not reverse the Ca²⁺-induced apoptosis of TL cells suggesting that calcineurin (22, 23) is not involved in the transduction of Ca²⁺-mediated apoptotic signals.

Several control experiments were performed to ascertain that FCM measurements detect apoptosis, rather than nonspecific cell death. Microscopic appearance of Ca²⁺-stimulated CD25⁻ TL and steroid-treated CD25⁺ TN and CD25⁻ TL cells, but not of Ca²⁺-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 Ca²⁺-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 Ca²⁺ or Mg²⁺. 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 Ca²⁺ in response to ionomycin. The analysis of Ca²⁺ fluxes in Fluo-3-loaded thymocyte subsets revealed, however, that both subsets are able to mobilize equally high Ca²⁺ 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,
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 \( \beta\)-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- subsets, 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\(^{hi}/\)an 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 others 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-) stages, 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.

References

1. Weiss, A., J. Imboden, D. Schoback, and J.D. Stobo. 1989. Role of T3 surface molecules in human T cell activation: T3-dependent activation results in an increase in cytoplasmic free calcium. Proc. Natl. Acad. Sci. USA. 86:4269.

2. Smith, C.A., G.T. Williams, R. Kingston, E.J. Jenkinson, and J.J.T. Owen. 1989. Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures. Nature (Lond.). 337:181.

3. Murphy, K.M., A.B. Heimberger, and D.Y. Loh. 1990. Induction by antigen of intrathymic apoptosis of CD4 \^{8} \wedge \text{TCR}^{b} \text{thymocytes in vivo}. Science (Wash. DC). 250:1720.

4. Shi, Y., R.P. Bissonnette, N. Parfrey, M. Szalay, R.T. Kubo, and D.R. Green. 1991. In vivo administration of monoclonal antibodies to the CD3 T cell receptor complex induces cell death (apoptosis) in immature thymocytes. J. Immunol. 146:3340.

5. Wylie, A.H. 1980. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature (Lond.). 284:555.

6. Raff, M.C. 1992. Social controls on cell survival and cell death. Nature (Lond.). 356:397.

7. Cohen, J.J. 1991. Programmed cell death in the immune system. Adv. Immunol. 50:55.

8. Payvar, F., D. DeFranco, G.L. Firestone, B. Edgar, O. Wrange, S. Okret, J.-A. Gustafsson, and K.R. Yamamoto. 1983. Sequence-specific binding of glucocorticoid receptor to MTV DNA at sites within and upstream of the transcribed region. Cell. 35:381.

9. Nikolić-Žugić, J. 1991. Phenotypic and functional stages in thymocyte development. Immunol. Today. 12:65.

10. Nikolić-Žugić, J., M.W. Moore, and M.J. Bevan. 1989. Characterization of the subset of immature thymocytes which can undergo rapid in vitro differentiation. Eur. J. Immunol. 19:649.

11. Nikolić-Žugić, J., and M.W. Moore. 1989. T cell receptor expression on immature thymocytes with in vivo and in vitro precursor potential. Eur. J. Immunol. 19:1957.

12. Nikolić-Žugić, J., and M.J. Bevan. 1988. Thymocytes expressing CD8 differentiate into CD4^{+} cells following intrathymic injection. Proc. Natl. Acad. Sci. USA. 85:8633.

13. Shen, F.-W. 1981. Monoclonal antibodies to mouse lymphocyte differentiation alloantigens. In Monoclonal Antibodies and T Cell Hybridomas. G. Hammerling, U. Hammerling, and J.F. Kearney, editors. Elsevier Science Publishers BV, Amsterdam. 25-31.

14. Scollay, R., A. Wilson, A. D'Amico, K. Kelly, M. Egerton, M. Pease, L. Wu, and K. Shortman. 1988. Developmental status and reconstitution potential of subpopulations of murine thymocytes. Immunol. Rev. 104:81.

15. Petrie, H.T., M. Pearse, R. Scollay, and K. Shortman. 1990. Development of immature thymocytes: initiation of CD3, CD4, and CD8 acquisition parallels down-regulation of the interleukin-2-receptor α-chain. Eur. J. Immunol. 20:2813.

16. Nicolleti, I., G. Migliorati, M.C. Pagliacci, F. Grignani, and C. Riccardi. 1991. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J. Immunol. Methods. 139:271.
17. Thornthwaite, J.K., E.V. Sugarbaker, and W.J. Temple. 1980. Preparation of tissues for DNA flow cytometric analysis. Cytometry. 1:229.

18. Andjelid, S., N. Jain, and J. Nikolić-Žugić. 1993. Ontogeny of fetal CD8\textsuperscript{+} thymocytes: expression of CD44 and CD25 and early expression of TCR α mRNA. Eur. J. Immunol. 23:2109.

19. Nunez, G., L. London, D. Hockenbery, M. Alexander, J.P. McKearn, and S.J. Korsmeyer. 1990. Deregulated Bcl-2 gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell lines. J. Immunol. 144:3602.

20. Takahama, Y., and A. Singer. 1992. Post-transcriptional regulation of early T cell development by T cell receptor signals. Science (Wash. DC). 258:1456.

21. Wilson, A., H.T. Petrie, R. Scollay and K. Shortman. 1989. Int. Immunol. 1:605.

22. Clipstone, N.A., and G.R. Crabtree. 1992. Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. Nature (Lond.). 357:695.

23. O'Keefe, S.J., J. Tamura, R.L. Kincaid, M.J. Tocci, and E.A. O'Neil. 1992. FK-506- and CsA-sensitive activation of the interleukin-2 promoter by calcineurin. Nature (Lond.). 357:692.

24. Strasser, A., A.W. Harris, and S. Cory. 1991. bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship. Cell. 67:889.

25. Sentman, C.L., J.R. Shutter, D. Hockenbery, O. Kanagawa, and S.J. Korsmeyer. 1991. bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. Cell. 67:879.

Abnormal differentiation of thymocytes in mice treated with CsA. Nature (Lond.). 336:176.

27. Jenkins, M.K., R.H. Schwartz, and D.M. Pardoll. 1988. Effects of CsA on T cell development and clonal deletion. Science (Wash. DC). 241:1655.

28. Vasquez, N., J. Kaye, and S.M. Hedrick. 1992. In vivo and in vitro clonal deletion of double-positive thymocytes. J. Exp. Med. 175:1307.

29. Ucker, D.S., J. Meyers, and P.S. Obermiller. 1992. Activation-driven T cell death. II. Quantitative differences distinguish stimuli triggering nontransformed T cell proliferation or death. J. Immunol. 149:1583.

30. Watanabe-Fukunaga, R., C.I. Brannan, N.G. Copeland, N.A. Jenkins, and S. Nagata. 1992. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature (Lond.). 356:314.

31. Shi, Y., J.M. Glynn, L.J. Guilbert, T.G. Cotter, R.P. Bissonnette, and D.R. Green. 1992. Role for c-myc in activation-induced apoptotic cell death in T cell hybridomas. Science (Wash. DC). 257:212.

32. Finkel, T.H., J.W. Kappler, and P.C. Marrack. 1992. Immature thymocytes are protected from deletion early in ontogeny. Proc. Natl. Acad. Sci. USA. 89:3372.

33. Finkel, T.H., J.C. Cambier, R.T. Kubo, W.K. Born, P. Marrack, and J.W. Kappler. 1989. The thymus has two functionally distinct populations of immature αβ\textsuperscript{+} T cells: one population is deleted by ligation of αβTCR. Cell. 58:1047.