Maturation Versus Death of Developing Double-Positive Thymocytes Reflects Competing Effects on Bcl-2 Expression and Can Be Regulated by the Intensity of CD28 Costimulation

David J. McKean, Catherine J. Huntoon, Michael P. Bell, Xuguang Tai, Susan Sharrow, Karen E. Hedin, Abigail Conley and Alfred Singer

*J Immunol* 2001; 166:3468-3475; doi: 10.4049/jimmunol.166.5.3468

http://www.jimmunol.org/content/166/5/3468

References

This article cites 49 articles, 26 of which you can access for free at:
http://www.jimmunol.org/content/166/5/3468.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2001 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Maturation Versus Death of Developing Double-Positive Thymocytes Reflects Competing Effects on Bcl-2 Expression and Can Be Regulated by the Intensity of CD28 Costimulation

David J. McKeans, Catherine J. Huntoon, Michael P. Bell, Xuguang Tai, Susan Sharrow, Karen E. Hedin, Abigail Conley, and Alfred Singer

Immature double-positive (DP) thymocytes mature into CD4+CD8+ cells in response to coengagement of TCR with CD28 costimulatory receptors, but the molecular basis for DP thymocyte apoptosis by TCR plus CD28 coengagement is not known. In the present study, we report that TCR plus CD28 coengagement does not invariably induce DP thymocyte apoptosis but, depending on the intensity of CD28 costimulation, can induce DP thymocyte maturation. We demonstrate that distinct but interacting signal transduction pathways mediate DP thymocyte maturation signals and DP thymocyte apoptotic signals. Specifically, DP maturation signals are transduced by the extracellular signal-related kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway and up-regulate expression of the antiapoptotic protein Bcl-2. In contrast, the apoptotic response stimulated by CD28 costimulatory signals is mediated by ERK/MAPK-independent pathways. Importantly, when TCR-activated thymocytes are simultaneously coengaged by both CD28 and CD2 receptors, CD28 signals can inhibit ERK/MAPK-dependent Bcl-2 protein up-regulation. Thus, there is cross-talk between the signal transduction pathways that transduce apoptotic and maturation responses, enabling CD28-initiated signal transduction pathways to both stimulate DP thymocyte apoptosis and also negatively regulate maturation responses initiated by TCR plus CD2 coengagement. The Journal of Immunology, 2001, 166: 3468–3475.

T cell development in the thymus involves the integration of signals transduced by multiple cell surface receptors and results either in thymocyte maturation or thymocyte apoptosis. A very large repertoire of structurally unique αβ Ag receptors are expressed first at the double-positive (DP; CD4⁺CD8⁺) stage of development, and signals generated by TCR ligation are required for further maturation of DP thymocytes to single-positive (CD4⁺ or CD8⁺) cells (1). Thymocyte development is programmed to selectively differentiate cells that express receptors capable of binding foreign peptides on self-MHC molecules while destroying potentially self-reactive cells. A large fraction of developing thymocytes (>95%) die as a result of this selection process (2). DP thymocytes that express TCR unable to bind intrathymic ligands die by neglect (stimulus-independent apoptosis). This selection is observed experimentally in animals that fail to express both MHC class I and class II TCR ligands, resulting in developmental arrest at the DP stage (3, 4). In contrast, negative selection is a stimulus-dependent apoptotic process. Thymocytes expressing potentially dangerous autoreactive TCR undergo apoptosis as a result of high-avidity interactions between the cell’s TCR and MHC plus self-peptide complexes (5, 6). DP thymocytes that receive appropriate signals from TCRs with low avidity for MHC complexes initiate maturation of DP cells to single-positive cells (positive selection).

It has been shown previously that TCR engagement is necessary but not itself sufficient to induce DP thymocytes to either mature or die (7, 8). Indeed, a variety of different thymocyte receptors (exemplified by CD2 but including CD5, CD24, CD49d, CD81, and TSA-1) have been identified that when coengaged with TCR can coinduce DP thymocyte maturation (9). In contrast, the costimulatory receptor CD28 is the only known surface receptor that when coengaged with TCR can costimulate DP thymocyte apoptosis (10). We have comparatively characterized the differentiation responses initiated in DP thymocytes as a result of TCR cross-linking with or without coengagement with CD28 and/or CD2. Both CD28 and CD2 receptors are expressed on all DP thymocytes and the ligands for both receptors are expressed by thymic stromal cells (11–13). Our results demonstrate that maturation and apoptotic responses are related but separable components in the thymocyte differentiation program. The nature and strength of the costimulatory response initiated by CD28 receptors can determine whether CD4⁺CD8⁻ thymocytes mature to CD4⁺CD8⁻ cells or undergo apoptosis. When signals are integrated from both CD28 and CD2 receptors in TCR-activated thymocytes, signals from the CD28 receptor can negatively regulate the responses initiated by the CD2 receptor.
Materials and Methods

Mice

Female C57BL/6 (B6) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). (Bcl-2 × B6)F1, mice were produced by crossing human Bcl-2-transgenic mice (driven by the lck proximal promoter) (14); provided by Dr. Stanley Korsmeyer) with B6 mice.

Abs and reagents

Anti-CD28 (mAb 37.51; Ref. 12) was purchased from BD Pharmingen (San Diego, CA) or purified from hybridoma culture supernatant with Affigel protein A MAPS II buffer kit (Bio-Rad, Hercules, CA). Anti-TCR-β (mAb H57-597; Ref. 15) and normal rabbit IgG also were purified from culture supernatant by Affigel affinity chromatography. Anti-CD2 (mAb RM2-5), anti-CD69 (mAb H12F3), anti-CD4 (mAb RL-172), and anti-CD8 (mAb 3-155) were purchased from BD Pharmingen, FITC protein A was purchased from Sigma (St. Louis, MO), and FITC anti-Bcl-2 (N-19) Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-Ip90 (calnexin) was described previously (16). PD98059 was purchased from Calbiochem (San Diego, CA).

Cell preparation and culture conditions

CD4⁺CD8⁺ DP thymocytes were purified from 4- to 6-wk-old female mice by panning on anti-CD8 (mAb 83-12-5)-coated plates as described previously (9). This purification procedure consistently produces thymocyte populations that are >96% DP cells. Purified DP thymocytes were cultured for 4–48 h at 37°C in a CO₂ incubator in culture medium consisting of RPMI 1640 medium supplemented with 5 × 10⁻³ M 2-ME, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS. Cells (4–5 × 10⁶/ml) were stimulated for various periods of time in 24-well plates that had been precoated by overnight culture (4°C) of purified Ab combinations in PBS (9). Stimulation/recovery assays were performed as described previously (9). Briefly, purified thymocytes were cultured in vitro for 18 h in Ab-coated wells (stimulation culture, day 1) and then transferred into recovery cultures in the absence of Ab stimulation for an additional 24 h (day 2). Cells were preincubated with PD98059 (50 μM, Calbiochem) for 30 min before stimulating on Ab-coated plates.

Phenotype analysis by flow cytometry

Cultured cells were incubated with saturating concentrations of FITC- or PE-labeled Abs in staining medium (HBSS, 0.5% BSA, 0.5% NaN₃) for 30 min at 4°C. Apoptotic cells were identified by staining with either FITC-annexin V or propidium iodide (PI). Labeled cells were analyzed using CellQuest software on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). All experimental results presented are representative of data obtained from at least three independent experiments.

Western blot analysis

Detergent lysates from cultured cells (2 × 10⁶ per sample) were analyzed on SDS-PAGE, transferred to an Immobilon membrane (Millipore, Bedford, MA), and blotted with anti-Bcl-2 (BD PharMingen) and protein A HRP (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Western blots were analyzed with an Ambis 4000 densitometer (Ambis, San Diego, CA).

Results

Identification and characterization of the molecular events responsible for signaling thymocyte development in vivo is complicated significantly by numerous receptor/ligand interactions between developing thymocytes and stromal cells in the thymic cortex. In contrast, purification of DP thymocytes away from all other thymic elements makes it possible in vitro to engage specific receptors on their surface and to assess the effect of their engagement on DP thymocyte fate. We previously have described a two-step culture system in which purified DP thymocytes, after being stimulated in the first culture, convert into CD4⁺CD8⁺ cells on transfer into nonstimulatory recovery cultures (9). In this two-step culture system, DP thymocytes are signaled to mature into CD4⁺CD8⁺ cells by coengagement of TCR with a variety of other surface receptors, which we have referred to as “coinducer molecules” (9). Importantly, engagement of surface TCR complexes is not by itself sufficient to stimulate maximal DP thymocyte maturation into CD4⁺CD8⁺ cells, nor is it sufficient by itself to stimulate DP thymocyte death (7, 8, 17). In this report, we designate receptors that drive maturation in TCR-activated DP thymocytes as coinducer receptors and receptors that drive apoptosis as costimulator receptors. Our analyses assess the molecular events induced by surface receptor coengagements that stimulate DP thymocyte maturation with those that stimulate DP thymocyte death.

Complex fate of DP thymocytes stimulated by coengagement of TCR and CD28 surface receptors

Because CD28 is the only known surface receptor that when coengaged with TCR stimulates DP thymocyte apoptosis, we assessed whether there would be conditions in which TCR plus CD28 coengagement might instead signal DP thymocyte maturation. We stimulated DP thymocytes with varying concentrations of anti-CD28 and/or anti-TCR mAbs and assessed their conversion into CD4⁺CD8⁻ cells. Even when DP thymocytes are stimulated with high concentrations of anti-TCR mAb (50 μg/ml), the TCR signals alone stimulate submaximal levels of maturation and little if any apoptosis. As reported previously (10), coengagement of TCR plus CD28 surface complexes on DP thymocytes with high doses of anti-CD28 mAb yielded few viable cells, and these few cells remained CD4⁺CD8⁻ (Fig. 1A). Remarkably, however, although CD2 coinduction incrementally increases maturation in a concentration-dependent manner, the ability of TCR plus CD28 engagement to stimulate DP thymocyte differentiation varied in a bimodal distribution with the concentration of anti-CD28 mAb used (Fig. 1, A and B). Coengagement of TCR plus CD28 surface complexes with low doses of anti-CD28 mAb did stimulate DP thymocytes to mature into CD4⁺CD8⁻ cells (Fig. 1A), and even lower concentrations of anti-CD28 (<1 μg/ml) stimulated progressively less thymocyte maturation in the absence of stimulus-dependent apoptosis (data not shown). Coengagement of TCR plus CD28 surface complexes with intermediate to high concentrations of anti-CD28 mAb stimulated progressively fewer DP thymocytes to mature (Fig. 1B) and more DP thymocytes to die (Fig. 1C) during the 2-day assay. Stimulation with either anti-CD28 or anti-CD2 alone elicited no detectable differentiation response (data not shown).

Because low-intensity TCR plus CD28 coengagement stimulated DP thymocyte maturation, we considered that high-intensity signals from TCR plus CD28 coengagement might initiate similar maturation signals in DP thymocytes except for the fact that the cells were also signaled to die. Consequently, we assessed TCR plus CD28 coengagement in DP thymocytes from Bcl-2-transgenic mice because Bcl-2 blocks TCR plus CD28-induced DP thymocyte death (10, 14) (Fig. 2A). We found that TCR plus CD28 coengagement with high concentrations of anti-CD28 was capable of inducing DP thymocyte maturation (Fig. 2B), but only in Bcl-2-transgenic mice, as high-intensity TCR plus CD28 coengagement induced wild-type B6 DP thymocytes to die (Fig. 2A). In Bcl-2 mice, the CD4⁺CD8⁻ thymocytes that matured in response to TCR plus CD28 engagement also expressed maturation markers CD69⁺ and CD5high at levels comparable to that observed in B6 CD4⁺CD8⁻ thymocytes stimulated by engagement of TCR plus CD2 (data not shown). Thus, high-intensity TCR plus CD28 coengagement does generate maturation signals that induce DP thymocytes to convert into CD4⁺CD8⁻ cells, but only if the signaled cells are prevented from undergoing apoptosis.

Interrelationship between DP thymocyte maturation signals and DP thymocyte apoptotic signals

Thus far, we have demonstrated that TCR-stimulated DP thymocytes are induced to mature by low-intensity CD28 signals but are induced to die by high-intensity CD28 signals. This observation raised the possibility that the signal transduction pathways mediating DP thymocyte maturation and apoptosis...
were interrelated in some fundamental way. To assess the interrelationship between DP thymocyte maturational and apoptotic signals, we performed all subsequent experiments with TCR plus CD2 coengagement, which is a potent inducer of DP thymocyte maturation, and high-intensity TCR plus CD28 coengagement, which is the only defined inducer of DP thymocyte apoptosis. The anti-TCR concentrations used in these experiments initiated maximum TCR-initiated thymocyte maturation responses.

Induction of endogenous Bcl-2 protein expression in signaled DP thymocytes and the role of the extracellular signal-related kinase (ERK)/mitogen-activated protein kinase (MAPK) signaling pathway

We began our assessment of the relationship between DP thymocyte maturation and apoptotic signals by examining their effect on up-regulation of antiapoptotic proteins in DP thymocytes from normal B6 mice. We found that intracellular Bcl-2 protein expression was induced in DP thymocytes by TCR engagement alone.
Anti-TCR plus a low concentration of anti-CD28 induced a small additional increase in the level of Bcl-2, and anti-TCR plus anti-CD2 further increased Bcl-2 expression levels (Fig. 3A). In contrast, TCR plus high-intensity CD28 coengagement failed to increase intracellular Bcl-2 protein expression beyond the minimal level induced by TCR engagement alone (Fig. 3A). Thus, DP thymocyte maturational signals induced by TCR plus CD28 coengagement up-regulate Bcl-2 protein expression, whereas DP thymocyte apoptotic signals induced by TCR plus high-intensity CD28 coengagement do not.

Because the ERK/MAPK pathway regulates DP thymocyte maturation during positive selection (18–20), we evaluated whether Bcl-2 up-regulation in DP thymocytes is stimulated similarly by the ERK/MAPK pathway. To assess this possibility, we used the drug PD98059 to block MAPK kinase (MEK) activity (21) in stimulated DP thymocytes. We found that PD98059 does indeed block Bcl-2 up-regulation in DP thymocytes activated by TCR plus CD2 coengagement (Fig. 3B). Thus, the ERK/MAPK pathway transduces TCR plus CD2 coengagement signals in DP thymocytes and up-regulates the antiapoptotic protein Bcl-2.

We next examined the effect of PD98059-mediated inhibition of MEK activity on DP thymocyte maturation and apoptosis. TCR plus CD2 and TCR plus CD28 coengagements both rapidly induce cell surface expression of CD69 (Fig. 4E), which identifies thymocytes committed to the thymocyte maturation program (22, 23). However, CD69+ DP thymocytes stimulated by TCR plus CD2 coengagement continue to mature into CD4+CD8− cells, whereas CD69+ DP thymocytes stimulated by TCR plus CD28 (high-intensity) coengagement subsequently undergo apoptosis. It can be seen that PD98059 inhibits both TCR plus CD2 (Fig. 4A) and TCR plus CD28 (Fig. 4B)-induced DP thymocyte maturation into CD69+ cells. However, PD98059 did not inhibit TCR plus CD28-induced apoptosis of CD69+ DP thymocytes as assessed either by annexin V staining (Fig. 4D) or PI staining (data not shown). Thymocytes treated with PD98059 and stimulated with TCR plus CD2 engagement did not exhibit a stimulus-dependent apoptotic response (Fig. 4C). Together these results demonstrate that the ERK/MAPK pathway transduces TCR plus CD28 and TCR plus CD2 coengagement signals but does not transduce TCR plus CD28 apoptotic signals. These results also demonstrate that maturation and apoptosis are independently regulated events stimulated by TCR plus CD28 coengagement.

Integration of signals from multiple coinducer receptors results in apoptosis rather than maturation

Our present results have demonstrated that the ERK/MAPK pathway transduces maturation signals but does not mediate DP thymocyte apoptotic signals. However, developing thymocytes interact in the thymus with stromal cells, and the outcome of the differentiation response is dictated by the integration of signals from multiple surface receptors. Thus, we evaluated Bcl-2 protein expression and cell survival after simultaneously coengaging TCR with both CD2 and CD28 surface receptors on DP thymocytes. Western blot analysis of Bcl-2 protein expression in stimulated DP thymocytes revealed that TCR engagement alone induced a low-level Bcl-2 protein expression (Fig. 5, A and B; compare lanes 1 and 2). However, unlike TCR plus CD2 coengagement, which further up-regulated Bcl-2 protein expression (Fig. 5, A and B, lane 3), TCR plus CD28 coengagement did not further up-regulate Bcl-2 expression (Fig. 5, A and B, lane 4). More importantly, simultaneous coengagement of TCR with both CD2 and CD28 receptors resulted in Bcl-2 protein levels comparable to that observed in DP thymocytes stimulated with TCR plus CD28 rather than the augmented levels found in thymocytes stimulated with TCR plus CD2. Thus, TCR plus CD28 coengagement actively inhibits Bcl-2 up-regulation by TCR plus CD2 (Fig. 5, A and B, compare lanes 3 and 5).

Because TCR plus CD28 coengagement actively inhibits Bcl-2 up-regulation stimulated by TCR plus CD2, it would be predicted that
TCR plus CD2 coengagement would not alter the fate of TCR plus CD28-signaled DP thymocytes. In fact, this is precisely what we observed in thymocytes simultaneously coengaged by TCR plus CD28 plus CD2. The number of DP thymocytes surviving stimulation with TCR plus CD28 of graded intensities was unaffected by the presence or absence of additional TCR plus CD2 coengagements (Fig. 5C). Thus, there is cross-talk between signals initiated by CD28 and CD2.

FIGURE 4. Inhibition of MEK blocks maturation but not apoptosis in TCR plus CD28-stimulated thymocytes. DP thymocytes were stimulated in vitro with anti-TCR with or without anti-CD2 or anti-TCR plus anti-CD28 for 4 h in the presence or absence of the MEK inhibitor PD98059. Thymocytes were analyzed by flow cytometry with PE-labeled anti-CD69, FITC-annexin V, and PI. The level of CD69 expression (mean fluorescence intensity; MFI) (A and B) was calculated on the fraction of cells identified as live differentiating thymocytes (CD69^+ annexin^-) (left). The fraction of differentiating apoptotic thymocytes (CD69^+ annexin^+) (C and D) recovered from the culture are described above (right). Fig. 4E shows a representative flow cytometric analysis of DP thymocytes stimulated with anti-TCR (H57 5 μg/ml) with or without anti-CD2 (10 μg/ml) or anti-CD28 (10 μg/ml) and stained with PE-labeled anti-CD69, FITC-annexin V, and PI. The CD69^+ thymocytes in the untreated sample represent in vivo-activated cells. Treatment with PD98059 did not affect the number of PI-negative cells in this assay.

FIGURE 5. Signal integration of CD28 and CD2 coinducer receptors assayed by measuring cell viability and the regulated expression of Bcl-2 protein. A, Bcl-2 Western blot analysis of DP thymocytes that were stimulated in vitro for 18 h with combinations of anti-TCR (5 μg/ml), anti-CD2 (10 μg/ml), and anti-CD28 (30 μg/ml). Cytosolic extracts from the stimulated cells were analyzed by Western blot developed with anti-Bcl-2 and then with anti-IP90 (loading control). B, Densitometric analysis of the Bcl-2 Western blot (shown in A). The relative levels of Bcl-2 expression are normalized to the IP90 loading control. C, The CD28-dependent apoptotic response dominates the CD2-dependent maturation response when thymocytes are coinduced with both anti-CD28 and anti-CD2. DP thymocytes were stimulated for 18 h in vitro with anti-TCR (5 μg/ml) with or without anti-CD2 (10 μg/ml) plus varying concentrations of anti-CD28. Viable thymocytes were identified by flow cytometry after staining with PI.

**Interrelationship between signaling pathways for DP thymocyte maturation and DP thymocyte death**

The present results demonstrate that coengagement of TCR with other surface receptors generate intracellular signals that result in either DP thymocyte maturation or apoptosis and that these two dramatically different outcomes are regulated by interrelated but distinct signaling pathways. Indeed, the results of the present study also provide a novel synthesis of the interactive intracellular events that are activated by coengagement of surface molecules on DP thymocytes and lead either to DP thymocyte maturation or cell death (Fig. 6).

T lymphocyte plasma membranes contain lipid rafts that are enriched in a variety of intracellular signaling molecules and cell surface coinducer molecules (24–27). TCR coengagement with coinducer molecules such as CD2, or TCR coengagement with CD28, promotes recruitment of TCR/CD3 complexes to lipid rafts (26) and promotes formation of an “immunological synapse” that is needed for efficient transduction of DP thymocyte maturational signals. However, in addition to DP thymocyte maturational signals, coengagement of TCR and CD28 also transduces proapoptotic signals in DP thymocytes that: 1) block ERK-mediated Bcl-2 up-regulation, and 2) lead to activation of intracellular caspases (10). Importantly, the intensity of these proapoptotic signals increase with increasing intensity of CD28 costimulation so that apoptotic predominates over maturation in DP thymocytes that have been stimulated by TCR plus CD2 plus CD28 high-intensity coengagements.

**Discussion**

Although distinct intracellular signaling pathways regulate maturation and apoptosis in developing DP thymocytes, the present study demonstrates that these two signaling pathways importantly interact with one another in developing DP thymocytes. We examined the interrelationship between DP thymocyte maturation and apoptotic signals as a consequence of observing that coengagement of TCR with CD28 costimulatory molecules could induce either DP thymocyte apoptosis or DP thymocyte maturation, depending on the intensity of CD28 costimulation. We found that DP thymocyte maturation signals, which are transduced by the ERK/MAPK pathway, also signal up-regulation of the antiapoptotic protein Bcl-2. In contrast, DP thymocyte apoptotic signals are independent of the ERK/MAPK pathway and actively inhibit up-regulation of Bcl-2. Active inhibition of Bcl-2 up-regulation provides a compelling explanation for why apoptotic signals predominate over maturational signals in developing DP thymocytes, a predominance that is critical for avoiding positive selection of autoreactive thymocytes during repertoire selection in the thymus.

To initiate the activation program in naive peripheral T lymphocytes, the TCR-MHC/peptide complex must interact for a period of time that is sufficient to initiate the appropriate receptor-proximal intracellular signaling events (28–31). Costimulatory signals lower the activation threshold of the T lymphocyte, permitting activation responses to suboptimal TCR-MHC interactions. In addition, failure to acquire appropriate costimulatory signals can result in T lymphocyte anergy rather than activation (32). Because costimulation is such an important component of the activation program in peripheral T lymphocytes, it is not surprising that similar signals contribute to regulating the thymocyte differentiation program. Although both naive peripheral T lymphocytes and DP thymocytes require “second signals” to initiate their TCR-initiated activation programs, the “second signals” stimulate phenotypically different responses in thymocytes as compared with mature T lymphocytes. Because peripheral T lymphocytes previously have undergone selection to remove self-reactive lymphocytes, strong activation signals resulting from engagement of TCR plus CD28 costimulatory receptors should initiate T cell growth, differentiation, and production of effector lymphocytes. In contrast, strong signals initiated by the same TCR and CD28 receptors in DP thymocytes occur as a result of stimulation by autoantigens and should elicit an apoptotic response. Although the stringency of the receptor-initiated requirements needed to stimulate opposing phenotypic events in DP thymocytes and peripheral T lymphocytes should be similar, the intracellular signaling mechanisms responsible for this apparent dichotomy are poorly understood.

During the last several years, considerable effort has been invested in characterizing the peptide ligands that are capable of stimulating positive and negative selection (33–37). Because these analyses have been done either in vivo or in fetal thymic organ culture, they have not addressed the potential contributions of coinduction signals that are provided by thymic stromal cells. To characterize the potential effects of coinduction responses, the DP thymocytes must first be separated from thymic stromal cells that express coinducer ligands. We have addressed this problem by using an in vitro thymocyte differentiation assay that involves stimulating purified DP thymocytes with maximum TCR activation signals plus varying amounts of Abs to defined cell surface costimulatory receptors (9). By using this in vitro assay, Cibotti et al. (9) showed that CD2, CD5, CD24, CD28, CD49d, CD81, and TSA-1 coinducer receptors could initiate the maturation program in DP thymocytes. Negative selection also can be regulated by multiple cell surface receptors. Punt et al. (10) identified two distinct death mechanisms in DP thymocytes. One required simultaneous engagement of TCR and CD28, whereas a second was initiated by TCR engagement in the absence of a costimulatory signal.

**FIGURE 6.** A model of engagement of TCR and CD2 and/or CD28 receptors produces interactive signals from lipid rafts that localize at the immunological synapse. Engagement of TCR plus CD2 initiates the activation of the ERK/MAPK pathway, which regulates both maturation and Bcl-2 production and can be inhibited by the MEK inhibitor PD98059. Strong signals from CD28 in TCR-activated DP thymocytes stimulate an apoptotic response. Although Bcl-2 is capable of inhibiting apoptosis, engagement of TCR plus CD28 stimulates signals that negatively regulate Bcl-2 production and stimulate apoptosis. Thus, CD28 engagement negatively regulates the CD2-inducible expression of the antiapoptotic Bcl-2 protein, and the CD28-dependent apoptotic response dominates the maturation response stimulated by CD2 coinduction.
and death was initiated by subsequent interaction with an undefined stimulus on APCs. Although the signaling mechanism responsible for the CD28-dependent apoptotic response remains uncharacterized, it has been shown to be caspase dependent and independent of death receptors Fas, TNFR (p55 and p75), and CD30 (10). CD5, CD28, and TNF receptors were reported to stimulate negative selection in fetal thymic organ culture (38); however, the phenotype of the targeted thymocytes was not identified. In addition, Kishimoto and Sprent (39) reported that receptors (CD5, CD43) that could not induce apoptosis in DP thymocytes could still induce apoptosis in semimature heat-stable Aghigh CD4+CD8+ thymocytes. Thus, different stages of thymocyte development may use different receptors to costimulate negative selection. The identification of redundant receptor-initiated costimulators of negative selection help explain why little or no defects in negative selection are observed in CD28−/−, CD43−/−, TNFR−/− or Fas-deficient lpr/lpr mice (40–42). A number of different T lymphocyte costimulatory receptors have been identified in various T lymphocyte subsets (43, 44). Because the mature T cell repertoire needs to be purged of cells reactive to the combination of self-Ags and costimulatory molecules expressed by professional APCs, it is likely that additional costimulatory receptors will be identified that regulate negative selection in thymocytes.

Previous studies have suggested a correlation between the expression of either proapoptotic or antiapoptotic Bcl-2 family members with stimulus-dependent apoptosis during thymocyte development (17, 45–49). These analyses were done on mixed cell populations stimulated to undergo apoptosis with various agonists. Data presented in this report demonstrate that TCR-activated DP thymocytes increase Bcl-2 production, and this level is increased further by engagement of the CD2 receptor. Because thymocytes from Bcl-2-transgenic mice are resistant to receptor-dependent and receptor-independent apoptosis (death by neglect), the TCR- and TCR plus CD2-inducible expression of Bcl-2 likely maintains thymocyte viability during maturation and enhances positive selection. Similar TCR- or CD2-coinducer-dependent increases in expression of Bcl-x or Bad were not observed by flow cytometry and Western blot analyses of DP thymocytes (data not shown).

Recent data demonstrate that an immunological synapse forms at the junction where a T cell engages its TCR by interacting with ligand on the surface of an APC (24–26). TCR cross-linking results in its redistribution into the lipid rafts that contain costimulatory receptors and intracellular signaling molecules (Fig. 6). Formation of this multireceptor structure promotes protein-protein interactions needed for TCR signal transduction. Engagement of costimulatory receptors enhances the receptor redistribution and clustering of rafts at the T cell-APC interface (26). The results presented in this report and others (9, 10) demonstrate that signals from different coinducer/costimulatory receptors (e.g., CD2 vs CD28) not only enhance thymocyte differentiation responses but can direct the outcome of the differentiation program in TCR-activated DP thymocytes. It has been suggested that costimulatory receptor signals merely amplify TCR-initiated responses rather than provide unique intracellular signaling responses (26). However, although signals from either CD2 or CD28 receptors can stimulate thymocyte maturation, the apoptotic response increases as the strength of the CD28 costimulatory signal increases. These results demonstrate that CD28 regulates apoptotic responses by mechanisms that are distinct from maturation events initiated by coinducer receptors such as CD2. It is possible that CD28 initiates unique signaling responses, in part, because it may not localize in the lipid rafts like other coinducer receptors (27).

Signals initiated from the combined engagement of TCR plus CD2 stimulate the ERK/MAPK pathway to regulate the thymocyte maturation response (Fig. 6). Inhibition of MEK (with PD9089) blocks DP thymocyte maturation stimulated by engagement of TCR plus either CD2 or CD28. These results are consistent with previous studies, which showed the importance of the ERK/MAPK pathway in thymocyte maturation (19–21). The Bcl-2 response that is induced by TCR plus CD2 also is completely inhibited in DP thymocytes treated with the MEK inhibitor PD98059. In PD98059-treated, TCR plus CD28-stimulated DP thymocytes, maturation is inhibited but apoptosis continues to occur in a CD28-dependent manner. Thus, the alternative outcomes of thymocyte differentiation, maturation, and apoptosis, can be independently regulated. Because there is no detectable TCR plus CD2-dependent apoptotic response in PD98059-treated thymocytes, CD2 coinducer signals do not initiate an apoptotic response that is masked by the inducible expression of Bcl-2.

Thymocyte development is not initiated by the ligation of the TCR and a single coinducer/costimulatory receptor. As a thymocyte migrates through the thymus, it can interact with many receptors. The outcome of the differentiation program (maturation, apoptosis) will be determined by the integration of signals initiated by multiple cell surface receptors. An example of this is observed when DP thymocytes are stimulated with the combination of anti-TCR, anti-CD2, and anti-CD28. The CD2-dependent increase in Bcl-2 is not observed in TCR-activated DP thymocytes stimulated by simultaneous engagement of both CD2 and CD28. Thus, CD28 signals can interfere with an antiapoptotic activation response initiated by CD2 coinduction. These results predict that CD28-initiated signals negatively regulate a kinase in the ERK/MAPK pathway.

Our results demonstrate that, as outlined in Fig. 6, the CD28 receptor can be coupled to multiple signaling responses in DP thymocytes that have received maximum TCR signals. At very low CD28 receptor engagement the ERK/MAPK pathway regulates thymocyte maturation as well as the expression of the antiapoptotic protein, Bcl-2. An ERK/MAPK-independent pathway becomes activated with higher levels of receptor engagement, and this pathway initiates apoptosis. Because CD28-induced signals negatively regulate the TCR plus CD2-induced expression of Bcl-2, there also is cross-talk between a CD28-dependent pathway and the ERK/MAPK-dependent pathway that regulates Bcl-2 expression. The relationship between the signals responsible for the apoptotic response and the signals that negatively regulate Bcl-2 expression has not been elucidated. However, because all DP thymocytes express CD28 and dendritic cells in the thymus cortical-medullary junction and cortex express high levels of B7-1 (CD80) and B7-2 (CD81), it is likely that the apoptotic response and the negative regulation of Bcl-2 are the physiologically relevant responses in thymocytes that have received maximal stimulation through the TCR. The net effect of these interactive events is that receptors that can regulate apoptosis dominate responses elicited from receptors that initiate maturation. Such a receptor hierarchy is appropriate if the goal of the thymocyte differentiation program is to eliminate cells that express Ag receptors with high affinity for self-Ags. Characterization of the interactive signals initiated from the TCR and multiple coinducer/costimulatory receptors will enhance our understanding of the molecular mechanisms responsible for regulating positive and negative selection during thymocyte development.

Acknowledgments
We thank Kenneth Offord (Mayo Clinic Biostatistics Research Center, Rochester, MN) for statistical analyses.


