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The Influence of the MAPK Pathway on T Cell Lineage Commitment

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Summary

During development, progenitor thymocytes differentiate into either CD4 or CD8 T cells, and this fate decision depends on the specificity of the T cell antigen receptor (TCR) for MHC class II or class I molecules. Based on the mechanisms of fate specification known for simple metazoan organisms, we sought to determine whether the extracellular signal-related kinases (ERKs) play a role in T cell differentiation and lineage commitment. Using a dominant gain-of-function mutant of the *erk2* gene, we show that differentiation into the CD4 lineage is favored. We also show that, conversely, the addition of a pharmacological inhibitor of the ERK pathway favors differentiation into the CD8 lineage. We present a quantitative selection model that incorporates these results as well as those of recent reports on the role of Notch in T cell lineage specification.

Introduction

There are two major steps in T cell development. The first is initiated by the rearrangement of the T cell antigen receptor (TCR) β-chain and the expression of the pre-T cell receptor (Godfrey et al., 1993; Groettrup et al., 1993; Dudley et al., 1994; Hoffman et al., 1996). This event provokes the cells to enter the cell cycle and differentiate from $CD4^-CD8^-$, double-negative (DN) thymocytes, into $CD4^+CD8^+$, double-positive (DP) thymocytes. The second step involves selection mediated by the TCR (reviewed by Robey and Fowlkes, 1994; Kisielow and von Boehmer, 1995). Subsequent to a productive TCR α-chain rearrangement, the DPs undergo either apoptosis or differentiation into mature T cells; there is evidence that the outcome is decided by the recognition of a specific peptide bound to major histocompatibility (MHC) molecules, and the difference is in the avidity or affinity of the interaction (Jameson et al., 1995). The intracellular signaling that specifies expansion, differentiation, and positive versus negative selection is poorly understood, but, depending on the experimental approach, evidence indicates that the Ras-to-MAPK (mitogen-activated protein kinases or extracellular signal-related kinases [ERKs]) pathway is critically involved (Alberola-Ila et al., 1996; Crompton et al., 1996; Swat et al., 1996).

A further aspect of this fate decision is that the cells differentiate into either CD4 or CD8 T cells depending on the TCR specificity for either MHC class II or class I molecules. Two possibilities have been suggested to explain the coordination of phenotype with MHC recognition (Chan et al., 1994a; Davis and Littman, 1994; Robey and Fowlkes, 1994). One is "instructional." That is, the recognition of a particular class of MHC molecules instructs the DPs to differentiate. How the signals from MHC class I and class II differ is unspecified, but recent experiments provide evidence for a quantitative instructional model (Itano et al., 1996; Matechak et al., 1996). The notion is that a relatively strong signal, usually accompanied by CD4–MHC class II interactions, instructs DP thymocytes to differentiate into $CD4^+CD8^-$ (CD4 single-positive [CD4SP]) T cells and that a weaker signal, usually accompanied by CD8–MHC class I interactions, results in $CD4^-CD8^+$ (CD8SP) T cells. Support for this mechanism comes from experiments that relied on the deletion or modification of coreceptors to provide more or less signaling to the DP thymocytes. A second class of models invoked to explain CD4 versus CD8 phenotypic differentiation have been termed "stochastic-selective" (Davis et al., 1993; Chan et al., 1994b). The notion is that DP thymocytes, which receive a signal through the TCR, randomly (stochastically) begin to differentiate into a CD4 or a CD8 T cell. A consequence of this is that one of the two coreceptors is down-regulated, and thymic maturation is completed only if the stochastic decision is consistent with the specificity of the TCR for either MHC class I or class II molecules.

Ultimately, an understanding of lineage commitment will depend upon establishing the receptor-mediated signaling pathways that alter nuclear transcription. Toward that end, recent work has shown that the cell surface receptor Notch may influence CD4 versus CD8 lineage decisions in a manner similar to the way it determines primary versus secondary cell fate of Drosophila melanogaster peripheral neurogenesis and Caenorhabditis elegans vulval fate differentiation (Robey et al., 1996). Although a mechanism of activation and a linear signaling pathway are known for the imaginal disc proneural clusters (Bailey and Posakony, 1995), the equivalent signaling cascade has not been established for mammalian Notch. Furthermore, it is not known, in any organism, how Notch signaling is related to the MAPK cascade.

A powerful tool in the establishment of signaling cascades important in development is the screen for hypermorphic gain-of-function mutations that suppress a genetic deficiency. Although the size of the genome and the generation time is such that we cannot readily carry out this sort of analysis in mice, we can take advantage of mutants isolated in flies and worms to understand the way a signaling network affects a mammalian developmental system. Using the mammalian equivalent of

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a gain-of-function mutation in Drosophila *erk-A*, we have established a role for ERK2 in the CD4 versus CD8 lineage decision in T cell development. We then supported the conclusions from these experiments by conducting further studies using a pharmacological agent to interrupt the activation of ERK.

Results

The Production of Transgenic Mice Expressing a Hypersensitive Form of ERK2

To study the role of ERK2 in thymocyte development, we expressed a hypersensitive form of ERK2 based on the Drosophila mutant *sevenmaker* (*rl*^{sem}) in transgenic mice. This mutant was isolated by a genetic screen for a dominant suppressor of *bride of sevenless* (*boss*), and it was found to arise from an aspartate-to-asparagine substitution at amino acid 334 in ERKA (Brunner et al., 1994). In addition to activating the *sevenless* pathway, leading to the formation of the R7 cells in the retina, *rl*^{sem} was found to activate the *torso* (*tor*) pathway, and it affects some, but not all developmental pathways mediated by the Drosophila homolog of the epidermal growth factor (EGF) receptor, DER. A mouse ERK2 homolog was produced by substituting an aspartate at 319 with an asparagine (referred to here as ERK2^{sem}), and it was shown to manifest a hypersensitivity due to delayed dephosphorylation (Bott et al., 1994; Ward et al., 1994). The baseline activity is equivalent to wild type, and so this is not a constitutively active but rather a hypersensitive form of ERK2.

The Myc-tagged mouse ERK2^{sem} was cloned into the pTEX expression vector, based on the human CD2 promoter/enhancer/locus control region (Lake et al., 1990; Zhumabekov et al., 1995), and the transcriptional cassette was used to produce transgenic mice (Figure 1A). Six lines of transgenic mice were produced, leading to 10 independent lines because of multiple incorporations. Using a probe from the human CD2 vector, five lines expressed detectable message in the thymus by Northern blots. Of these, three expressed detectable levels of ERK2^{sem} using an ERK2-specific polyclonal antisera (C-14). The line expressing the highest level of ERK^{sem} protein, sem20, had approximately 10 copies of the transgene, as estimated by Southern blotting, and was bred for analysis.

ERK2 could be detected as a 42 kDa protein on Western blots from thymus and lymph node cells (Figure 1B). Since ERK2^{sem} contained a Myc tag in our experiments, it ran with slightly reduced mobility on SDS–polyacrylamide gel electrophoresis (PAGE), as seen in the thymus and lymph nodes from sem20 mice. We estimate that the transgene was expressed at a level no greater than 20% of endogenous ERK2.

Activation with phorbol myristic acid (PMA) is known to stimulate phosphorylation of ERK2 through Raf-1 (Kolch et al., 1993; Marquardt et al., 1994; Qiu and Leslie, 1994), and this is revealed by the decreased mobility of phospho-ERK. In thymus and lymph node cells from sem20 mice treated with PMA, there was an easily detectable phosphorylated form of ERK2^{sem} (Figure 1B). A lower-mobility phosphorylated form of the wild-type

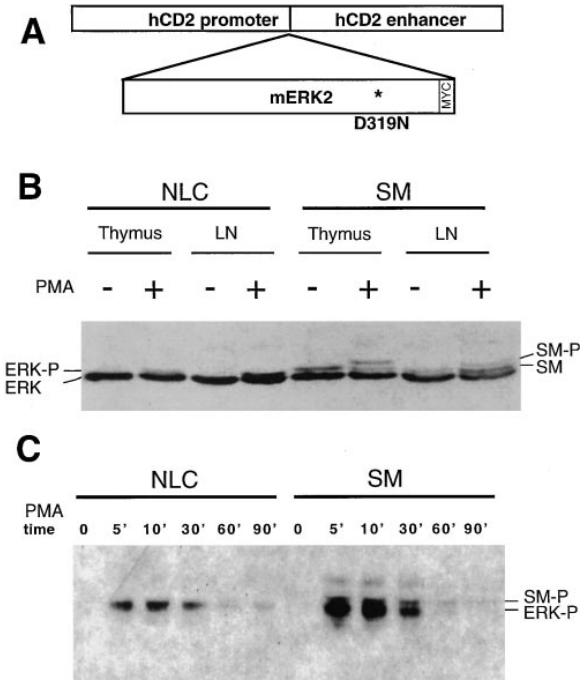

Figure 1. ERK2^{sem} Is Expressed and Can be Activated in Lymphocytes

(A) Representation of the pTEX-SM targeting construct used in the generation of ERK^{sem} (SM) transgenic mice (see Experimental Procedures).

(B) Cells (5×10^6) from the indicated organs were incubated at 37°C for 10 min in the presence or absence of 5 ng/mL PMA. The cells were then lysed and immunoblotted using anti-ERK2 (C-14). LN, lymph node; NLC, normal littermate control.

(C) Thymocytes were stimulated with 5 ng/mL PMA in Hank's balanced salt solution at 37°C for the indicated times. Whole-cell lysate (20 μg) was immunoblotted with anti-phospho-MAPK.

ERK2 was also detected, although the the phosphorylated and unphosphorylated forms did not entirely resolve (see also below, Figure 5). Compared with wild-type ERK2, a greater proportion of ERK2^{sem} was phosphorylated.

Using an antibody specific for phospho-ERK, we further tested the time course of ERK2 activation. As shown in Figure 1C, treatment of thymocytes with 10 ng/ml of PMA caused the appearance of phospho-ERK2 within 5 min. This form of ERK2 was detected for at least 30 min and was essentially undetectable after 60 min. Using thymocytes from sem20 mice, there was a very similar time course of ERK2 appearance; however, there was a substantial increase in the levels of phospho-ERK2. It appeared that both endogenous and ERK^{sem} were phosphorylated to higher levels, even though extracts were adjusted to 20 μg protein/well. We note that no background phospho-ERK2 was detectable in the absence of PMA.

These data indicate that the transgenic ERK2^{sem} is hypersensitive, though expressed and activated in manner very similar to that of endogenous ERK2. Although previous data indicated that dephosphorylation of ERK^{sem} was delayed compared to controls (Bott et al., 1994; Ward et al., 1994), the difference is probably

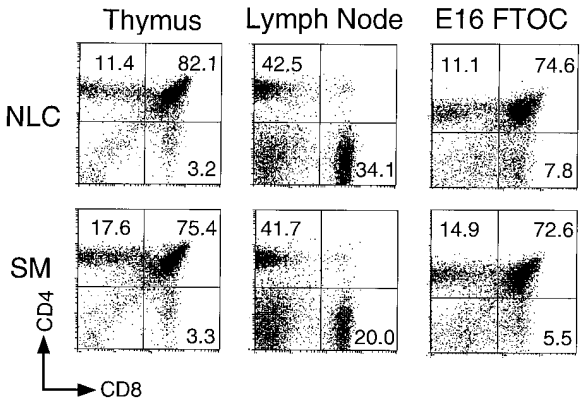

Figure 2. ERK^{sem} Alters Thymus and Lymph Node Subsets

Cells from sem20 (SM) or normal littermate control (NLC) mice were stained with anti-CD4-PE and anti-CD8-Tricolor. Numbers represent the percentage of live cells in the indicated quadrant. Ten thousand events are shown. E16 FTOC, cells after 5 days of culture starting at embryonic day 16.

too subtle to detect based on the time points measured. Kinase assays were not carried out since the Myc tag prevents immunoprecipitation with anti-ERK2 antibody. We therefore could not directly compare the kinase activity of the transgenic ERK2^{sem} with endogenous ERK2; however, PMA-mediated phosphorylation of ERK2 correlates well with activity (Marquardt et al., 1994).

The Expression of ERK^{sem} Alters T Cell Development

To investigate the role of ERK2 in thymic development, we examined the proportion and absolute numbers of cells in each of the thymic subsets characterized by the expression of CD4 and CD8. An exemplary experiment is depicted in Figure 2. Compared to normal littermate controls, there was an increased proportion of $CD4^+CD8^-$ thymocytes (CD4SP) and a coordinate decrease in the percentage of $CD4^+CD8^+$ (DP) thymocytes. The percentages of $CD4^-CD8^-$ (DN) and $CD4^-CD8^+$ (CD8SP) thymocytes were essentially unchanged, and there were no changes in the CD3 profiles in any of the subsets. The lymph node T cells showed a different effect: the percentage of CD4 T cells was unchanged, whereas the percentage of CD8 T cells was, on average, 67% of normal (Table 1).

The total cellularity of the thymi in sem20 mice was an average of 68% that of littermate controls, although there was no change in the total number of cells residing in the pooled inguinal, brachial, mesenteric, and submaxillary lymph nodes (Table 1). A compilation of the data confirms the results shown in Figure 2; the percentage of CD4SP thymocytes was significantly greater in sem20 mice compared with littermates, to a highly significant level by Student's t test ($P < 0.0001$). The difference in CD8 T cells in the lymph nodes also was highly significant ($P < 0.0005$). Thus, the expression of ERK^{sem} caused an alteration in the proportion of cells in the CD4 and CD8 lineages, although in adult mice, the increased proportion of CD4SP thymocytes is reflected in a decreased proportion of CD8 lymph node T cells.

We also wished to determine how the expression of ERK2^{sem} would affect the development of T cells in fetal thymic organ cultures (FTOCs). In young adult mice the populations reflect a steady state that is subject to homeostatic feedback mechanisms (Berg et al., 1989), and a high rate of apoptosis and engulfment (Surh and Sprent, 1994). On the other hand, in FTOCs the subsets represent a wave of maturation, which to some extent reflects the rate of development. As shown in Figure 2, 5-day FTOCs from embryonic day 16 (E16) sem20 mice showed skewing of thymocyte populations, such that there was a consistent increase in the proportion of CD4SP cells and a decrease in the proportion of CD8SPs compared to normal littermate controls. In the E16 FTOCs, the number of cells and the percentage in each subset were variable from experiment to experiment. However, the ratio of CD4SP to CD8SP cells was revealing: we found that the ratio of CD4SP to CD8SP for sem20 mice was 2.3 ± 0.38 ($n = 6$) and that for wild-type mice it was 1.6 ± 0.37 ($n = 5$) ($P < 0.02$).

To examine the effect of ERK^{sem} on negative selection, we examined superantigen-mediated deletion of SP thymocytes and lymph node T cells. The percentage of SP thymocytes and lymph node T cells that expressed Vβ11 is a measure of the deletion mediated by MMTV-8, 9, 17 (Scherer et al., 1993), whereas total Vβ8-bearing T cells are not altered by any of the MMTV proviruses in the strains tested. As shown, there was no difference in MMTV-mediated deletion of Vβ11-bearing T cells (Table 1). This outcome agrees with the work of Perlmutter and his colleagues in that the MAPK pathway affects positive but not negative selection (Alberola-Ila et al., 1995, 1996; Swan et al., 1995).

The Effects of ERK2^{sem} on T Cell Development in the Presence of a Class II–Specific Transgenic TCR

Since ERK^{sem} activity is inducible, presumably it will depend on signal transduction originating from ligation of the TCR (Izquierdo et al., 1993). Therefore we sought to examine the development of T cells in TCR transgenic

Table 1. Lymphocyte Subpopulations in *sevenmaker* Transgenic Mice

		No. of Cells ($\times 10^7$)	% CD4	% CD8	% Vβ8 SP	% Vβ11 SP
Thymus	NLC	9.5 ± 4.2	10.7 ± 1.4	3.1 ± 0.8	16.0 ± 0.6	2.1 ± 0.3
	SM	6.5 ± 2.6	$\underline{14.0 \pm 2.3}$	3.3 ± 1.0	16.4 ± 1.0	2.2 ± 0.2
Lymph node	NLC	2.7 ± 0.9	38.9 ± 4.1	25.0 ± 5.8	19.3 ± 0.4	2.6 ± 0.6
	SM	2.5 ± 0.9	38.8 ± 4.5	$\underline{16.9 \pm 3.3}$	19.4 ± 0.6	2.4 ± 0.4

Data are represented as means ± standard deviation. NLC, n = 16; SM, n = 18.
Underlining indicates SM values significantly different (Student's T test, $P < 0.0005$) from NLC.

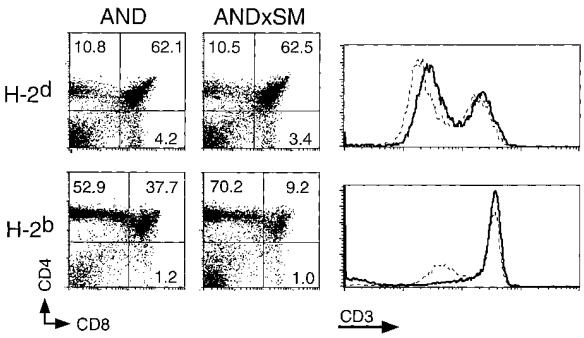

Figure 3. Alteration of Thymic Subsets in AND × sem20 (AND × SM) Double-Transgenic Mice Is MHC Haplotype–Dependent

Thymocytes were triple stained with anti-CD4-PE, anti-CD8-Tricolor, and anti-CD3-FITC. Numbers represent the percentage of cells in the indicated quadrant. Ten thousand events are shown. Dashed lines, AND normal littermate control; solid lines, AND × SM.

mice in which we could manipulate receptor recognition. The AND strain of mice has a transgenic TCR that is specific for pigeon cytochrome c (PCC) 88–104. sem20 mice were crossed with AND mice and bred to express different MHC molecules that do or do not support positive selection: H-2^b (A^b-E^o) and H-2^d (A^d-E^d).

In the AND-H-2^d nonselecting mice, ERK^{sem} had essentially no effect on the subsets of thymocytes. The 10% CD4 population resulted from dual expression of transgenic and endogenous TCR α-chains, according to flow cytometry analysis (data not shown) and the observation that AND-H-2^dRag-$2^{-/-}$ mice have virtually no CD4SP thymocytes (Lerner et al., 1996). In contrast, in the presence of TCR recognition associated with positive selection, there was a significant effect. Compared to AND-H-2^b mice, in double-transgenic AND × sem20-H-2^b mice there was a significant drop in thymus cellularity, a decrease in the proportion of DPs, and an increase in the proportion of CD4s. The small population of CD8SPs was unchanged (Figure 3 and Table 2).

This finding reinforces the notion that the transgenic $ERK2^{sem}$ influences the outcome of thymic development in response to TCR-mediated interactions. The CD3 expression profiles reflect the thymus subsets, in that DP thymocytes express an intermediate level of CD3 and SP thymocytes express it at a high level. The DN cells are composed of immature and mature thymocytes, and at least the mature DN thymocytes generally express high levels of CD3 in TCR transgenic mice. The reason that there appears to be no distinct CD3-intermediate population in the AND × sem20-H-2^b mice is that the DP population is small.

Interruption of the MAPK Pathway

To investigate further the role of ERK in T cell development we took advantage of a newly discovered compound, PD98059, from Parke Davis (Alessi et al., 1995; Dudley et al., 1995). This compound has been shown to inhibit the phosphorylation of MEK1 and MEK2 by upstream activators such as c-Raf and thus far appears to be highly selective. To determine the dose-response characteristics in lymphocyte cultures, we first titrated PD98059 into anti-CD3–stimulated $CD4^+$ lymph node T cell cultures from sem20 or littermates. As depicted in Figure 4A, PD98059 inhibited the proliferative response of both populations to about 14% of the maximum at a concentration of 25 μM. This concentration of PD98059 was also effective in antigen-stimulated cultures. Lymph node T cells from AND mice were stimulated with varying amounts of PCC 88–104 peptide in the presence and absence of 25 μM of PD98059. As shown, PD98059 shifted the dose-response curve by almost 100-fold to higher concentrations of added peptide antigen (Figure 4B). This result shows that MEK activation is essential for antigen-induced T cell proliferation. This finding is not consistent with the observation that a catalytically inactive form of MEK1 does not inhibit T cell proliferation (Alberola-Ila et al., 1995).

To demonstrate the effectiveness of PD98059 in blocking the activation of MEK and ERK, we first examined a proliferative response induced by the addition of PMA. A result that we did not anticipate was that thymocytes from sem20 but not wild-type mice proliferated with the addition of PMA alone. Since wild-type lymphocytes usually require an additional stimulus, such as an increase in cytoplasmic calcium, this finding indicates that proliferation of thymocytes can be mediated by ERK activation above the level that can be achieved by PMA stimulation in wild-type cells (Figure 5A). This proliferation was effectively inhibited by the addition of 17.5 μM PD98059. Since PMA has been shown to activate Raf−1 indirectly through protein kinase C (Kolch et al., 1993) and since cells transgenic for ERK^{sem}, but not wild-type cells, were activated, the inhibition by PD98059, as an inhibitor of MEK activation, is consistent with the canonical MAPK phosphorylation cascade of Raf-1→MEK→ERK (Howe et al., 1992; Kyriakis et al., 1992; Marshall, 1994). As a way of showing that the MEK inhibitor is effective in blocking ERK2 activation,

Table 2. Thymocyte Subpopulations in AND × SM TCR Transgenic Mice

MHC Haplotype		No. of Cells ($\times 10^7$)	% DP	% CD4	% CD8
H-2^d	AND	2.3 ± 0.3	56.7 ± 8.6	11.4 ± 1.4	5.8 ± 2.1
	AND × SM	2.4 ± 1.5	60.2 ± 3.2	10.4 ± 0.9	4.5 ± 1.7
H-2^b	AND	8.2 ± 4.0	35.5 ± 7.7	54.6 ± 8.7	1.3 ± 0.4
	AND × SM	3.1 ± 1.6	17.6 ± 3.4	66.2 ± 2.9	1.7 ± 0.7

Data are represented as means ± standard deviation. AND-H-2^d, n = 3; AND × SM-H-2^d, n = 4; AND-H-2^b, n = 13, AND × SM-H-2^b, n = 13. Underlining indicates AND × SM values significantly different (Student's T test, $P < 0.005$) from AND controls.

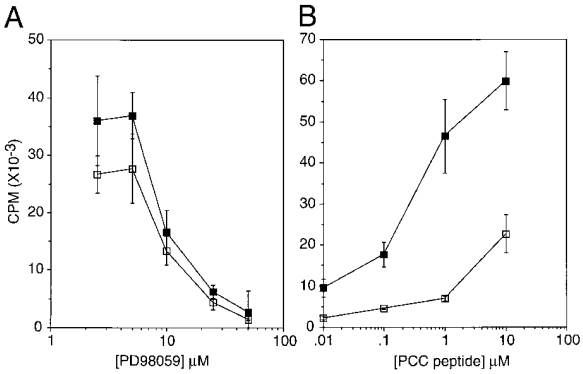

Figure 4. MEK Activation Is Required for TCR and Peptide-Mediated Proliferation

(A) Lymph node cells from a sem20 (SM) mouse (filled squares) or a normal littermate control (open squares) mouse were stimulated with 10 ng/well 145–2C11 (anti-CD3) and the indicated concentration of PD98059 MEK Inhibitor. Proliferation after 3 days was determined by the incorportion of 1 μCi of [^{3}H]thymidine.

(B) AND-H-2^b lymph node cells were cultured with mitomycin C–treated B10.A splenic antigen-presenting cells and the indicated concentrations of PCC peptide in the presence (open squares) or absence (filled squares) of 25 μM PD98059.

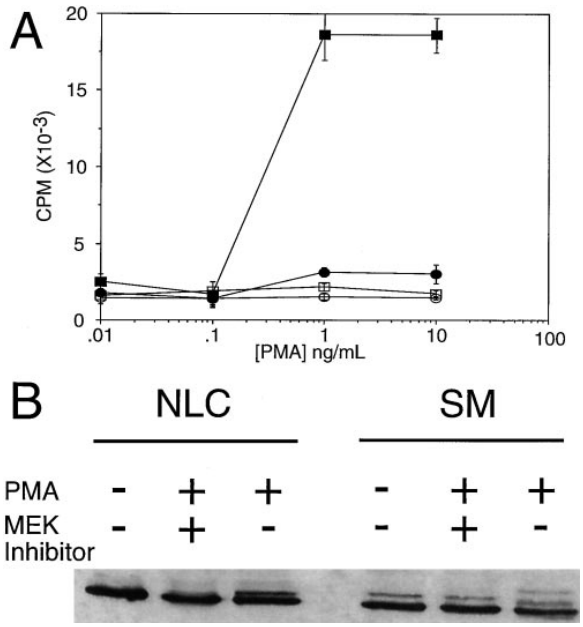

Figure 5. MEK Inhibitor Blocks Thymocyte Proliferation and ERK Phosphorylation

(A) Thymocytes from normal littermate control (NLC) mice (circles) or sem20 (SM) mice (square) were treated with varying concentrations of PMA (filled symbols) or PMA and 17.5 μM PD98059 (open symbols). Proliferation was measured after 3 days in culture by the incorporation [^{3}H]thymidine.

(B) Thymocytes were incubated at 37°C for 1 hr in the presence or absence of 30 μM PD98059 then for an additional 10 min in the presence or absence of 10 ng/mL PMA. Whole-cell lysates from 5 × 10^6 cells were immunoblotted with anti-ERK2 (C-14).

PD98059 was shown to inhibit the PMA-stimulated appearance of phospho-ERK2 in both wild-type and sem20 thymocytes (Figure 5B).

The Effect of Inhibiting ERK Activation on Thymic Development

The requirement for ERK activation in T cell development was examined by adding PD98059 to FTOCs from either E14 or E16 fetal mice. E14 thymi are composed entirely of early, progenitor DN cells, whereas E16 thymi have a population of cells that have already differentiated into DP thymocytes and have many fewer DN precursors. Organs were cultured for 5 days in the presence and absence of 30 μM PD98059, and the subsets present at the end of culture were analyzed by flow cytometry.

Since differentiation from E14 includes the proliferation step in thymic development, cell recoveries are highly dependent on the extent of expansion. For the E14 FTOCs, the average cellular recovery in the presence of PD98059 was 37% that of untreated cultures (Figure 6), implying that expansion of thymocytes depends in part on ERK activation. The possibility also exists that ERK is necessary for survival and that the decrease in cellularity is the result of accelerated apoptosis. Since this drop in cellularity was not seen in E16 cultures (Figure 7), the requirement for survival would have to be specific for the DN-to-DP transition. In addition, there was no obvious decrease in cell viability in FTOCs from sem20 mice at the end of culture; however, this issue is under further investigation.

The flow cytometry profile of a representative E14 FTOC is depicted in Figure 6. In untreated cultures the resulting cells are mainly DPs (81.8%). In cultures with 30 μM PD98059, the percentage of cells in the DP subset was reduced to slightly greater than 50% of controls, and this is seen both in the percentage of cells gated for CD4$^+$CD8$^+$ expression and in the percentage of cells that expressed a characteristic intermediate level of CD3. Considering the decrease in overall recovery, the absolute number of DPs in PD98059-treated organ cultures was therefore less than 20% that of controls. The inhibition of differentiation from DN to DP is thus not complete, but it is consistent with the magnitude of the effect of PD98059 on the proliferation of antigen-activated T cells.

In the wild-type cultures there were very few mature SPs, presumably reflecting the fact that the cells from 5-day E14 FTOCs have only just completed differentiation. Surprisingly, there was a large population of CD8SP cells in E14 cultures treated with PD98059, and 60% of these cells expressed high levels of CD3. Further analysis of these CD3 high cells has shown that a large number of the CD8SP cells expressed low levels of heat-stable antigen (data not shown), an indication that the cells are mature, antigen-responsive T cells (Ramsdell et al., 1991). Since this population was not seen in experiments initiated with E16 thymi (see below), we conclude that inhibition of ERK activation before the differentiation step to DP thymocytes dramatically promotes CD8SP thymocytes. Within the CD4SP population from wild-type mice there was a small but significant population of CD3^{hi} cells that was missing in cultures containing PD98059.

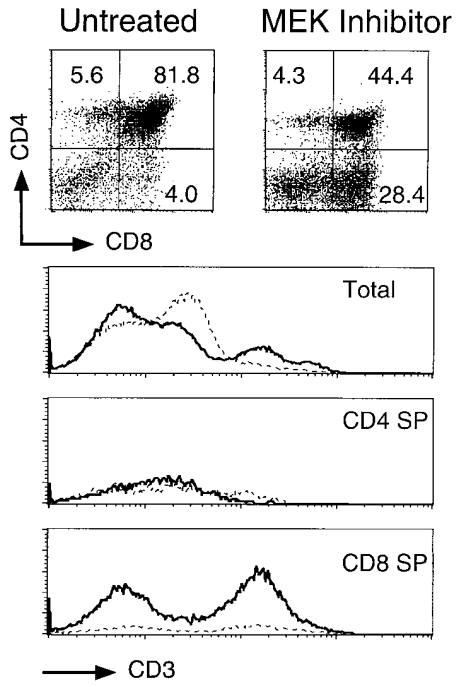

Figure 6. ERK Is Not Required for Transition to CD8 SP

E14 thymi were cultured for 5 days in the presence or absence of 30 μM PD98059 MEK inhibitor. Thymocytes were triple stained with anti-CD4-PE, anti-CD8-Tricolor, and anti-CD3-FITC. Numbers represent the percentage of live cells in the indicated quadrant. Histograms represent CD3 expression of total or SP thymocytes. Dashed lines, thymi cultured in medium alone; solid lines, thymi cultured with 30 μM PD98059. The average cell recovery ($\times 10^{-5}$) was 6.7 ± 0.6 for controls (n = 7) and 3.5 ± 2.2 for PD98059-treated cultures (n = 6).

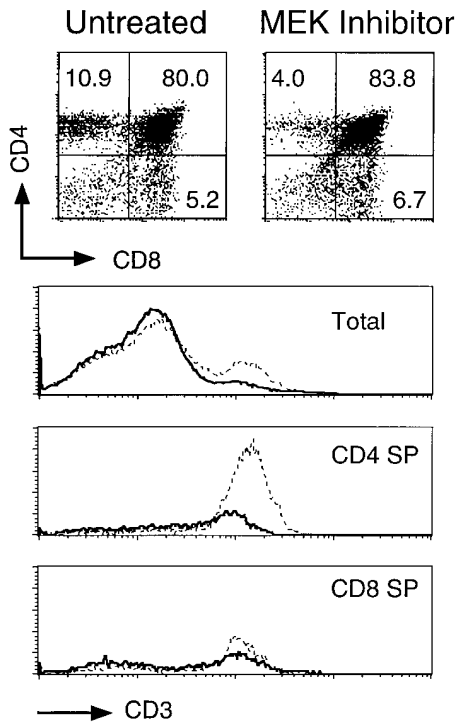

Figure 7. ERK Is Required for Maturation from DP to CD4 SP

E16 thymi were cultured for 5 days in the presence or absence of 30 μM PD98059 MEK inhibitor. Thymocytes were triple stained with anti-CD4-PE, anti-CD8-Tricolor, and anti-CD3-FITC. Numbers represent the percentage of live cells in the indicated quadrant. Histograms represent CD3 expression of total or SP thymocytes. Dashed lines, thymi cultured in medium alone; solid lines, thymi cultured with 30 μM PD98059. The average cell recovery ($\times 10^{-5}$) was 6.4 ± 3.7 for controls (n = 5) and 11.1 ± 3.5 for PD98059-treated cultures (n = 7).

The thymi from E16 mice begin with a large population of DPs, no SPs, and many fewer DN precursors compared to E14 thymi (data not shown). These cultures provide a way to examine the selection step and the transition from DP to SP in the absence of effects on the early expansion in the DN-to-DP transition. Of note, we saw enhanced cellular recovery in cultures treated with PD98059, but whether this reflects increased survival or expansion is not yet known (Figure 7). We also found a diminished number of mature thymocytes, a feature most easily seen by comparing the number of $CD3^{hi}$ cells (Figure 7). The important point is that in these cultures, we consistently found a reversal in the CD4:CD8 ratio caused by a diminishment in the proportion of CD4SPs and an increase in the proportion of CD8s. This result substantiates the alteration in lineage commitment seen in E16 FTOCs from sem20 mice.

Discussion

In this report we used genetic and pharmacological methods to show that ERK activation affects thymocyte selection and especially promotes commitment to the CD4 phenotype. We discuss the influence of ERK on early maturation and positive selection and comment on the possibility that both ERK and Notch play important roles in lineage commitment.

The Effect of ERK Activation on Early Expansion and Transition of Thymocytes from DN to DP

To analyze the effects of the inhibition of ERK activation, we carried out experiments in which E14 thymus lobes were cultured in the presence and absence of a MEK inhibitor, PD98059. All of the cells in these cultures begin as early DN thymocytes, and so we can examine the expansion and differentiation of thymocytes from DN to DP. Here we found that inhibition of ERK activation partially inhibited the expansion and differentiation of DN to DP cells. Considering that 25 μM PD98059 was sufficient to inhibit T cell proliferation and ERK phosphorylation only partially, and since drug availability in organ cultures may be diminished compared to suspension cultures, we interpret these data to support the notion that expansion and early differentiation are dependent on ERK activation. This is consistent with the results of Crompton et al. (Crompton et al., 1996), in which the expression of a dominant interfering form of MEK by retroviral expression in FTOCs caused inhibition of expansion. It is also consistent with the studies of Swat et al. (1996), in which an activated form of Ras promoted expansion in the absence of TCR rearrangements. In experiments using a combination of dominant interfering forms of Ras and MEK, Alberola-Ila et al.

(1996) did not see inhibition at this stage. These discrepancies have not yet been resolved, although the predominance of evidence favors the idea that ERK activation is involved in some aspects of survival, expansion, and differentiation at this stage of T cell development.

The Effect of ERK Activation on Positive Selection and Lineage Commitment

In sem20 mice there was a statistically significant increase in the percentage of cells that differentiated to be CD4SP thymocytes. This was supported by the observation that the CD4SP subset was enhanced in AND $\times$ sem20-H-2^b mice, in which the entire population of thymocytes was subjected to positive selection. Although ERK activation may have an overall effect on positive selection, this analysis with a gain-of-function hypersensitive form of ERK2 shows that ERK activation selectively promotes CD4SP maturation. We argue that this is actually an imbalance in the proportion of CD4 and CD8 T cells, based on four other observations. First, there was a highly significant decrease in the number and percentage of CD8 T cells in the lymph nodes of adult sem20 mice. Second, there was an increase in CD4SP cells and a decrease in CD8SP cells in E16 FTOCs from sem20 mice. This is more representative of the rate of maturation since it reflects the first wave of differentiated cells, and presumably it is influenced less by homeostatic mechanisms. Third, in E14 FTOCs treated with PD98059, there appeared a large population of CD8SP CD3^{hi} thymocytes, whereas the population of CD4SP CD3^{hi} thymocytes was undetectable. Fourth, in E16 FTOCs treated with PD98059 there was again an decrease in the proportion of CD4SP thymocytes and an increase in the proportion of CD8SP thymocytes.

Using both genetic and pharmacological devices, we conclude that positive selection and lineage commitment are regulated at least in part by activation of ERK2. In the work cited above, Alberola-Ila et al. (1996) showed that dominant interfering forms of Ras or MEK and especially the combination can inhibit positive selection and the differentiation of DPs to SPs. Although they did not note an effect on lineage commitment, in their data from mice with a catalytically inactive form of MEK-1, the ratio of CD4 to CD8 cells in adult thymi appeared to depend partly on the level of MEK–dominant negative expression (Alberola-Ila et al., 1995). In the latter study it is possible that the interfering form of MEK expressed was relatively ineffective in the inhibition of the ERK activation, a possibility consistent with its lack of effect on T cell proliferation. The interfering form of Ras certainly affected several downstream pathways, so the effects of p21H-rasN17 may be pleiotropic and more difficult to interpret. In the present studies we used two entirely different ways of altering the amount of activated ERK. While PD98059 may have effects beyond the inhibition of MEK, we propose that the analysis of ERK^{sem}, an inducible, hypersensitive mutant expressed at a low level, is a particularly incisive means of analyzing the role of ERK2 in T cell development. As mentioned above, *rl*^{sem} affects some but not all developmental processes mediated by the EGF receptor, implying that ERK^{sem} shows specificity for particular differentiation events. A combination of these two avenues of investigation provides a consistent view of ERK as a control point for thymocyte selection and as an arbiter of lineage commitment.

The maturation of CD4SP thymocytes in AND-H-2^d mice was not entirely predictable. As stated above, expression of A^d and E^d is not compatible with the differentiation of AND thymocytes (Lerner et al., 1996). The cells that do manage to differentiate have reduced levels of the transgenic Vα11, resulting from the expression of a second endogenous TCR α-chain. Nonetheless, presumably there is still a TCR-mediated selection that was not apparently influenced by ERK^{sem}. Whether this reflects the timing of selection or other influences of this genetic cross is not yet understood.

A Quantitative Instructional Mechanism for Lineage Commitment: ERK and Notch

Previous studies have provided evidence that lineage commitment can be explained in part by the magnitude of the signal received. In the one study, replacement of the CD8 cytoplasmic tail with that of CD4 caused the selection of thymocytes bearing an MHC class I–specific transgenic TCR to differentiate into CD4SPs (Itano et al., 1996). The cytoplasmic tail of CD4 is known to bind p56^{lck} more avidly than the cytoplasmic tail of CD8, and thus these cells were thought to get an abnormally strong coreceptor signal. Conversely, AND TCR transgenic thymocytes that expressed an MHC class II–specific TCR became CD8SPs in the absence of CD4 (Matechak et al., 1996). In this case, the absence of a coreceptor signal changed the lineage from CD4 to CD8. Both of these studies were interpreted to indicate that "strength" of the signal determined the lineage, and the strength was determined by signaling through the coreceptor and through the TCR.

To understand the mechanisms involved in fate determination it is useful to turn to developmental processes in simple organisms. There is a compelling analogy between T cell differentiation and that described for the adoption of primary, secondary, and tertiary fates in vulva development in C. elegans. In normal wild-type development, three cells receive an "inductive signal" from the anchor cell, and these three cells adopt the primary or secondary phenotypes of vulva cells. Genetic screens for abnormal vulval development (Ferguson et al., 1987) identified a series of mutants that were ordered through epistasis into a growth factor–activated (LIN-3) (Hill and Sternberg, 1992), receptor tyrosine kinase (RTK) (LET-23) (Aroian et al., 1990) pathway leading to ERK (SUR-1, MPK-1) activation (Lackner et al., 1994). Constitutive or enhanced activation along this canonical Ras-mediated pathway (Han and Sternberg, 1990) results in more than three vulval precursor cells (primary or secondary fates) characterized as Multivulva, whereas diminished signaling within this pathway results in fewer than three vulval precursor cells, that is, a greater number of cells that adopt the tertiary epithelial cell fate and a Vulvaless phenotype (Sundaram and Han, 1996). The adoption of a secondary cell fate by the outer two cells, P5.p and P7.p, requires a "lateral" signal through LIN-12 (analogous to Drosophila Notch) (Artavanis-Tsakonas et

al., 1995). Cells that do not get either of these signals adopt the tertiary fate of epithelia. Studies of LIN-3 overexpression suggest that the activation of the MAPK pathway causes supernumerary vulva cells with the primary fate and prevents adoption of the secondary fate (Katz et al., 1995). On the other hand, a hypermorphic gain-of-function mutation in *Lin-12* causes all six cells to adopt a secondary fate (Greenwald and Seydoux, 1990). In addition, mosaic studies in which the P5.p and P7.p cells have a *let-23* deficiency show that these cells can adopt the secondary fate in the absence of LET-23 signaling (Koga and Ohshima, 1995). Thus, the adoption of a primary, secondary, or tertiary fate depends on the amount and perhaps the timing of signaling through the MAPK and Notch pathways (Sternberg and Horvitz, 1989). Differentiation depends on an initial MAPK signal, whereas lineage specification depends on the strength of the MAPK signal and the presence of a Notch signal.

Similarly, the Notch (Artavanis-Tsakonas et al., 1995) and the MAPK pathways (Chang et al., 1994) control many aspects of Drosophila development, including retinal differentiation. For example, the cell fate specification of the R7 cell requires signaling through the *sevenless*-encoded receptor tyrosine kinase and activation through Ras and the MAPK pathway (Hafen et al., 1994; Zipursky and Rubin, 1994; Wassarman et al., 1995). Notch expression can modify this fate specification such that expression of an activated form of Notch under control of the *sevenless* promoter (*sev-Nact*) gives rise to a rough-eye phenotype, a phenotype similar to the weak activation of the MAPK pathway (Fortini et al., 1993). The concept for both vulva development in C. elegans and retinal development in Drosophila is that the balance of MAPK (inductive) and Notch (lateral) activation is important in primary versus secondary fate specification.

Recent compelling studies by Robey et al. (1996) provide evidence that Notch can also function to direct lineage commitment in thymocytes (Robey et al., 1996). The transgenic expression of an activated, intracellular form of Notch during T cell development favored the formation of CD8 T cells over CD4 T cells. Thus, there is a striking similarity between the lineage specification in T cell development and the basic principles of fate specification worked out using classic genetics. The experiments presented here show that ERK is required for differentiation to the CD4 lineage and that the strength of the signal determines the number of cells that adopt the primary fate versus the second fate. Based on the work of Robey et al. (1996), this selection may be modified by the presence of a strong signal through Notch. In thymus subsets, Notch is expressed at highest levels in DN thymocytes, lowest levels in DP thymocytes, and intermediate levels in SP thymocytes (Hasserjian et al., 1996). A possibility is that ERK activation may down-regulate the expression of Notch on developing DN thymocytes and thus interrupt Notch signaling. A weaker signal through the TCR or the coreceptor may be sufficient for differentiation but insufficient to down-regulate Notch. This notion is consistent our observation that inhibition of MEK during the DN-to-DP transition results in large CD8SP $CD3^{hi}$ population in part consisting of $\alpha\beta$ T cells (Figure 6). In the presence of the MEK inhibitor, late DN cells that have completed TCR β-chain rearrangements may adopt the CD8 $\alpha\beta$ phenotype perhaps without a transition through the DP phenotype. This may be analogous to an experiment in C. elegans in which the level of LIN-12 can be titrated such that the absence of an inductive (anchor) cell causes the P6.p cell to adopt a secondary phenotype, whereas the presence of an anchor signal causes the P6.p cell to adopt a primary phenotype (Sternberg and Horvitz, 1989).

As implied by Robey et al. (1996), we propose that the CD4 lineage should be referred to as the primary cell fate and the CD8 lineage the secondary cell fate. What we think of as the process of positive selection results from the inductive signal, and lineage specification may depend on a signal through Notch-1 and on the strength and timing of the inductive signal. These concepts definitely suggest a quantitative instructive mechanism for lineage commitment, although how the MAPK and Notch pathways are differentially activated by the TCR, CD4, and CD8 coreceptors remains to determined. Within this instructional mechanism of fate determination, there could be a stochastic component. This would originate from the probability that a thymocyte receives a strong signal early in development in order to activate ERK and down-regulate Notch. With time and the absence of sufficient ERK activation, Notch may initiate a positive feedback loop that results in differentiation toward the CD8 phenotype; this differentiation would continue if the TCR is class I specific but would abort for a lack of coreceptor signaling if the TCR is class II specific. The extensive genetic analyses already carried out in Drosophila and C. elegans provide many opportunities for further elaboration of these pathways in T cell development.

Experimental Procedures

Mice

Mice were generated, bred, and maintained at the University of California, San Diego animal facilities. Timed matings were set up by placing one male in a cage with two females for 16 hr. The day after the male was removed was considered day 1 of gestation. AND mice are transgenic for TCR α- and β-chains that confer specificity for PCC as described (Kaye et al., 1989, 1992).

Generation of Transgenic Mice

pEFD319NErk2 (Bott et al., 1994) was cut with NcoI and SpeI. Ends were filled using Klenow. This was ligated into pTEX that had been linerized with XhoI, blunted with Klenow, and treated with calf intestinal phosphatase. The resulting plasmid was cut with KpnI and SalI to liberate the *sevenmaker* transgene and purified over a sucrose gradient. This was injected into C57BL/6J $\times$ BALB/c F1 single-cell embryos at a concentration of 2 μg/ml. Founders were backcrossed to C57BL/6J.

Flow Cytometry and Antibodies

One million cells were washed in phosphate-buffered saline containing 2% fetal calf serum and 0.1% sodium azide and stained using anti-CD4-phycoerythrin (PE) (Caltag) and anti-CD8-TRICOLOR (Caltag) and anti-CD3-fluorescein isothiocyanate (FITC) (Pharmingen). Events were collected prior to analysis on a FACScan (Becton-Dickinson). Collection and analysis were performed using CellQuest software (Becton-Dickinson). New analysis gates were generated with each experiment to compensate for changes in forward scatter or side scatter as well as antibody staining intensity.

Western Analysis

Cells were lysed in Triton X-100 lysis buffer (0.5% Triton X-100, 10 mM Tris [pH 8.0], 150 mM NaCl, 200 μM sodium vanadate, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride). Next, 5×10^6 cell equivalents or 20 μg total cell lysate were separated by 12% SDS-PAGE and transferred to Protran membrane (Schleicher and Schuell). Membrane was blocked in TBST (Tris-buffered saline, 0.1% Tween-20) with 5% nonfat milk and incubated with anti-ERK2 (C-14, Santa Cruz Biochem) or anti-phospho-MAPK (New England Biolabs) in TBST, 5% milk. Blots were then incubated with goat anti-rabbit-horseradish peroxidase (Southern Biotechnology Associates) secondary antibody and developed using enhanced chemiluminescence (Amersham).

Fetal Thymic Organ Culture

Thymic lobes were harvested on E14 or E16 and placed on Transwell filters (Costar). Thymi were cultured in Iscove's modified Dulbecco's medium (supplemented with 10% fetal calf serum, glutamine, and antibiotics) with or without 30 μM PD98059 (New England Biolabs) at 37°C, 5% CO_2 for 5 days. Medium was replaced every 24 hr. Lobes were strained through nylon mesh to release thymocytes. Viable cells were counted by propidium iodide exclusion.

Proliferation Assays

$CD4^+$ lymph node cells were obtained by complement-killing total lymph node cells using 3.168 (anti-CD8) and low-toxicity rabbit complement (Cedarlane). Lymph node cells were added at 5×10^4 per well in EHAA medium (Gibco–BRL) supplemented with 10% fetal calf serum. Total thymocytes were added at 4×10^5 per well in EHAA. In experiments in which anti-CD3 was added, indicated concentrations of purified 145–2C11 were bound to plates pretreated with 10 μg/mL goat anti-hamster antibody (Southern Biotechnology Associates). Assays were pulsed with 1 μCi [^{3}H]thymidine (New England Nuclear) for the final 8–12 hr of culture.

AND TCR transgenic lymph node cells were collected and added at 2×10^4 cells/well in complete EHAA. After depletion of erythrocytes, mitomycin C–treated splenocytes from B10.A mice were added to culture at 5×10^5 cells/well as a source of antigen-presenting cells. PCC peptide 88–104 was added at the indicated final concentrations. Assays were pulsed with 1 μCi [^{3}H]thymidine for the final 8–12 hr of culture. In experiments containing MEK inhibitor, PD98059 (New England Biolabs) was added at 25 μM or the indicated concentration.

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