

Commitment to the CD4 Lineage Mediated by Extracellular Signal-Related Kinase Mitogen-Activated Protein Kinase and Lck Signaling¹

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The development of T cells results in a concordance between the specificity of the TCR for MHC class I and class II molecules and the expression of CD8 and CD4 coreceptors. Based on analogy to simple metazoan models of organ development and lineage commitment, we sought to determine whether extracellular signal-related kinase (Erk) mitogen-activated protein (MAP) kinase pathway signaling acts as an inductive signal for the CD4 lineage. Here, we show that, by altering the intracellular signaling involving the Erk/MAP kinase pathway, T cells with specificity for MHC class I can be diverted to express CD4, and, conversely, T cells with specificity for MHC class II can be diverted to express CD8. Furthermore, we find that activation of the src-family tyrosine kinase, p56^{lck} is an upstream mediator of lineage commitment. These results suggest a simple mechanism for lineage commitment in T cell development. *The Journal of Immunology*, 1999, 163: 6598–6605.

Two different models have been suggested to explain the process of T cell lineage commitment, including the association of TCR specificity and the appropriate expression of the CD4 and CD8 coreceptors (1). The major tenet of the stochastic/selective model is that there is no specificity to the signal received as a result of MHC class I recognition vs MHC class II recognition. The proposal is that, upon TCR/MHC interaction, one coreceptor randomly becomes down-regulated and further survival depends upon continuous MHC interactions with both the TCR and the CD4 or CD8 coreceptor. Cells expressing the correct coreceptor survive, whereas cells that express a coreceptor not in concert with the specificity of the TCR do not. Alternatively, mechanisms that include an MHC-specific signal to guide lineage commitment in accordance with TCR specificity fall under the rubric of instructional models.

Oddly, there is evidence in support of both mechanisms. An analysis of MHC-deficient (class I[−] class II[−]) mice showed that, by the criterion of CD4-CD8 expression levels, no differentiation past the CD4⁺CD8⁺ double positive (DP)³ step occurs. Notably, in MHC class I⁺ class II[−] mice, a population of immature CD4 thymocytes (CD4⁺CD8^{low}) arose. On the assumption that these cells were precursors to mature CD4 single positive T cells, the idea was put forth that MHC class I or II molecules can give an initial nonspecific signal to begin the process of positive selection (2). Similarly, in the absence of MHC class I, but the presence of

MHC class II, there was evidence for an immature CD8 population (2, 3). Further analysis indicated that the above assumption may be too simplistic (4, 5). A second line of experimentation showed that, in some cases, constitutive, transgenic expression of a coreceptor could permit the appearance of T cells with a specificity not matched to coreceptor expression (6–8). In other words, a transgenic coreceptor could “rescue” T cells that expressed a coreceptor not in concert with the TCR specificity. In other experiments along this line, there was no rescue seen (1), but, in any event, the rescued T cells were always few in number. These results may be consistent with a mechanism whereby there is an initial stimulation that causes cells to initiate differentiation, but this differentiation is not strictly random and is influenced by the signaling resulting from MHC class I or class II recognition (7).

Indeed, other experiments support the notion that there are signals that direct the differentiation of uncommitted thymocytes along one or another pathway. The use of Abs directed to the TCR in organ cultures causes a bias toward the CD4 lineage, except in one case where it causes a bias toward the CD8 lineage (9–11). There are no reports of balanced CD4 and CD8 maturation that should come from a mechanism based strictly on stochastic differentiation and selection. Other work showed that the signals required for CD4 and CD8 commitment appear to be asymmetric (12). Recent data looking at the signaling molecules that drive differentiation are also consistent with the notion that the nuances of signal transduction influence lineage commitment. We have shown that a hypersensitive form of extracellular signal-related kinase (Erk)-2 biases differentiation toward the CD4 lineage, and inhibition of the Erk-2 activation pathway using a pharmacological inhibitor biases differentiation toward the CD8 pathway. The deletion of *Csk*, a src family regulator, causes thymocytes to differentiate toward the CD4 lineage exclusively and in the complete absence of TCR expression (13). In addition, a deficiency in *c-Cbl*, a regulator of T cell signaling, promotes CD4 and not CD8 maturation (14). Finally, either activated Notch or Bcl-2 overexpression appears to promote differentiation to the CD8 lineage (15–17).

A simplistic view of thymocyte development is that TCR and coreceptor interactions with MHC class I or class II molecules signal cells to differentiate. The quality of the signal as well as other receptor-counter receptor interactions influence whether the

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³ Abbreviations used in this paper: DP, double positive; Erk, extracellular signal-related kinase; MAP, mitogen-activated protein; MEK, MAP/Erk kinase; SOP, sensory organ precursor; HSA, heat stable Ag; FTOC, fetal thymus organ culture; BrdU, 5-bromo-2'-deoxyuridine.

incipient T cell differentiates toward the CD4 or CD8 lineage (or dies as a manifestation of negative selection). The quality of the signal appears to depend on the strength of signaling through src family kinases and ultimately the activation of mitogen-activated protein (MAP) kinases such as Erk-2. Nonetheless, the initial signal may provide only an inductive bias, and it is possible that further signaling could be required to provide proofreading as means to substantially increase the accuracy of a lineage decision. This could be described as an instructional-selective model. It is consistent with much of the available evidence, and it also conforms to general schemes of lineage commitment worked out for organ development in simple metazoans (18). In this report, we provide evidence that CD4 and CD8 lineage commitment can be subverted by altering the strength of MAP kinase pathway signaling. Furthermore, we provide evidence that this signaling can originate from $p56^{lck}$ activation.

Materials and Methods

Mice

Mice were bred and maintained at the University of California at San Diego animal facilities. Timed matings were set up by placing 2–3 females with one male for 16 h. The day the male was removed was considered day 0 of gestation. Erk2^{sem} mice are transgenic for Erk2D319N (19). AND mice are transgenic for V α 11 and V β 3, which confers specificity for pigeon cytochrome c (PCC) (20, 21). OT-1 mice are transgenic for V α 2 and V β 5, conferring specificity for the OVA peptide SIINFEKL and were a gift from Michael Bevan (22). $p56^{lck}$ -F505 mice were a gift from Roger Perlmutter (23).

Fetal thymus organ culture

Thymus lobes were harvested on E16 and placed on Transwell filters (Costar, Cambridge, MA). Thymuses were cultured in IMDM (supplemented with 10% FCS, glutamine, sodium pyruvate, nonessential amino acids, 2-ME, and antibiotics) in the presence or absence of indicated concentrations of PMA, ionomycin, and PD98059 (New England Biolabs, Beverly, MA) at 37°C, 5% CO₂ for 5 days unless otherwise indicated. Medium was replaced every 48 h. Lobes were strained through nylon mesh to release thymocytes. Viable cells were counted using the flow cytometer.

Flow cytometry and Abs

Thymocytes were washed in PBS containing 2% FCS and 0.1% sodium azide. Cells were surface stained using combinations of anti-CD4 PE, anti-CD8 Tricolor (Caltag, South San Francisco, CA), anti-V α 11-FITC (PharMingen, San Diego, CA), anti-V α 2-FITC (PharMingen), and anti-HSA-biotin (PharMingen) + Streptavidin-APC (PharMingen). BrdU analysis was performed by adding 25 μ M BrdU (Sigma, St. Louis, MO) to the culture medium for 16 h before harvest. Staining was performed following the protocol of Carayon and Bord (24). Anti-BrdU-FITC was obtained from Becton Dickinson (Mountain View, CA). Events were collected before analysis on a FACScan (Becton Dickinson) or FACScalibur (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 Ab-staining intensity.

Results and Discussion

Erk Pathway inhibition increases CD8SP in AND TCR transgenic thymocytes

We have shown that fetal thymuses from normal mice cultured in the presence of PD98059, a specific inhibitor of MAP/Erk kinase (MEK), have a marked decrease in CD4SPs, but no substantial decrease in CD8SPs (19). To show the time course of the effects of MEK inhibition, we added PD98059 to fetal thymic organ cultures (FTOCs) at different stages of development (Fig. 1). The percentage of recovered CD4SP-TCR β -chain high thymocytes was plotted vs the day of fetal life at the initiation of a 5-day culture. As shown, the percentage of CD4s always exceeded that of CD8s in FTOCs treated with vehicle alone whereas, in the presence of 30 μ M PD98059, the percentage of CD8SPs exceeded that of

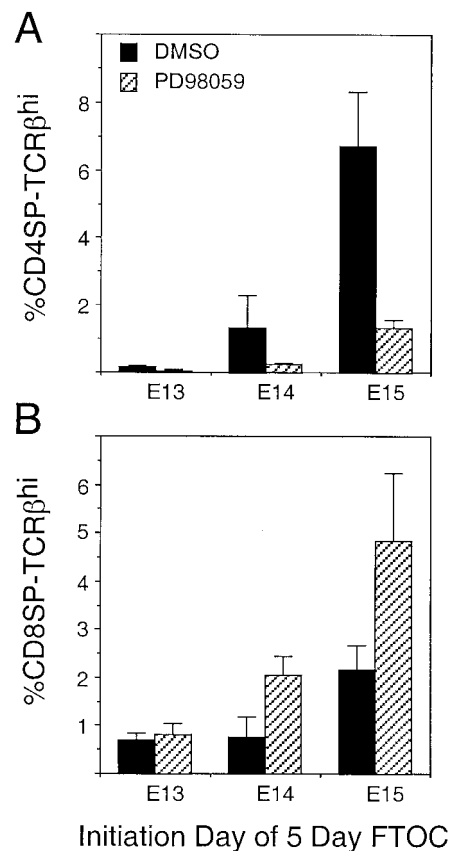


FIGURE 1. MEK inhibition selectively inhibits CD4SP maturation. FTOCs were initiated at the indicated day of embryonic life and cultured for 5 days with 30 μ M PD98059 or vehicle alone (DMSO). A, The values correspond to the percentage of CD4SP-TCR β -chain high cells (B), or the percentage of CD8SP-TCR β -chain high cells.

CD4SPs at every point. This experiment confirms our previous results (19) and demonstrates that the increased CD8SPs are $\alpha\beta$ T cells. It conflicts to some extent with the results of one subsequent report (25), but, as discussed below, it is consistent with a very recent report (26).

In cultures initiated before day E15, the recovery of thymocytes in the presence of PD98059 is diminished to between 20–30% of controls. This is because the inhibition of MEK is very effective in eliminating the proliferation and differentiation that gives rise to DPs (19); for this reason, a comparison of the actual number of recovered cells is difficult. As the day of culture initiation progresses, a substantial number of thymocytes have made the transition to the DP stage, and the recoveries are less affected to the point where recoveries are actually increased in day 16 cultures. In the series of d15 experiments presented in Table I, the number of CD4SPs recovered was greater than the number of CD8SPs in

Table I. CD4 and CD8 subsets present after MEK inhibition^a

	DMSO Control	30 μ M PD98059
Total recovery	865 $\pm$ 160	506 $\pm$ 140
CD4 ⁺ CD8 ⁺	666 $\pm$ 130	333 $\pm$ 130
CD4SP-TCR β ^{high}	57.5 $\pm$ 15.1	6.9 $\pm$ 3.5
CD8SP-TCR β ^{high}	18.9 $\pm$ 6.2	22.9 $\pm$ 2.0

^a Total number of recovered live cells ($\times 10^{-3}$) from individual organ cultures of E15 thymuses cultured for 5 days ($n = 4$ thymuses per group).

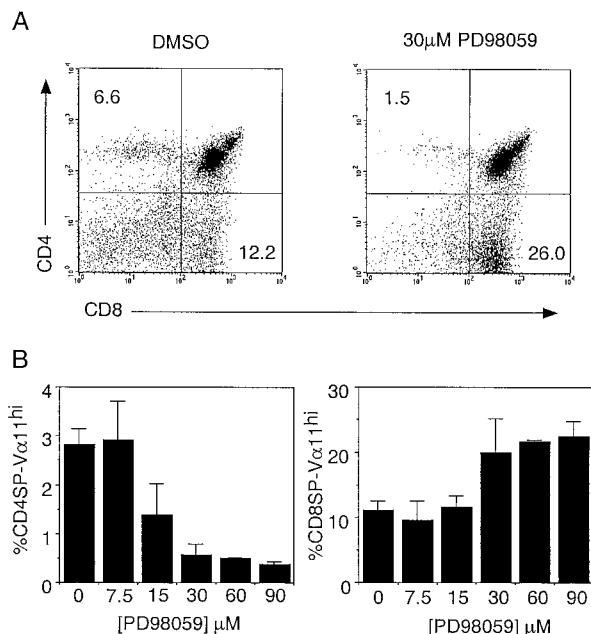


FIGURE 2. MEK inhibition increases CD8SP in MHC class II-specific thymuses. E16 thymuses from AND TCR transgenic mice were cultured for 5 days in the presence of the indicated concentration of PD98059. Thymocytes were stained for expression of CD4, CD8, and Vα11. *A*, A flow cytometric profile of a typical experiment using 30 μM PD98059. *B*, Histograms representing the percentage of Vα11⁺CD4SP cells (*left*) and Vα11⁺CD8SP cells (*right*).

control cultures, whereas the reverse was true of cultures treated with PD98059. Although the mean number of CD8SPs found with the addition of PD98059 was greater than that of controls, the variation precluded achieving statistical significance. At least under these conditions, we found that CD4SP differentiation was preferentially or even exclusively inhibited, but from this experiment we cannot tell whether class II-specific cells that failed to mature into CD4SPs changed their fate to become CD8SPs.

To determine whether MHC class II-specific precursors can be redirected to differentiate into CD8SPs, we analyzed the development of T cell precursors expressing a receptor known to be specific for MHC class II molecules. AND transgenic mice express TCR α- and β-chain genes that encode a TCR specific for pigeon cytochrome *c* and H-2E^{k,b} molecules. In the presence of H-2^b or H-2^k, there is a very large population of CD4SPs and virtually no CD8SPs that express Vα11 at high levels (20, 21). However, we note that, in FTOCs from AND-H-2^b mice, there is an unusual population of CD8s with highly variable levels of CD8 (Fig. 2A). We have seen this with other MHC class II-specific TCR transgenic mice as well. Nonetheless, thymuses from AND TCR transgenic mice were cultured in the presence or absence of 30 μM PD98059 and analyzed for the expression of CD4, CD8, and Vα11 (Fig. 2A). Clearly, there is a diminished percentage of CD4SPs and a large increase in the percentage of CD8SPs. In a titration of PD98059, there was a decreased proportion of Vα11^{high}CD4SPs and coordinately increased proportion of Vα11^{high}CD8SPs (Fig. 2B). Importantly, the number of CD4SP decreased from 9713 ± 3099 in mock-treated cultures to 2718 ± 1484 in cultures treated with 30 μM PD98059, whereas the number of CD8SP increased from 38,647 ± 12,118 to 87,813 ± 13,621, respectively. The conversion of class II-specific precursors to the CD8 lineage represents a demonstration that lineage commitment is obliged by the strength of MEK, and by inference, Erk activation. We surmise that

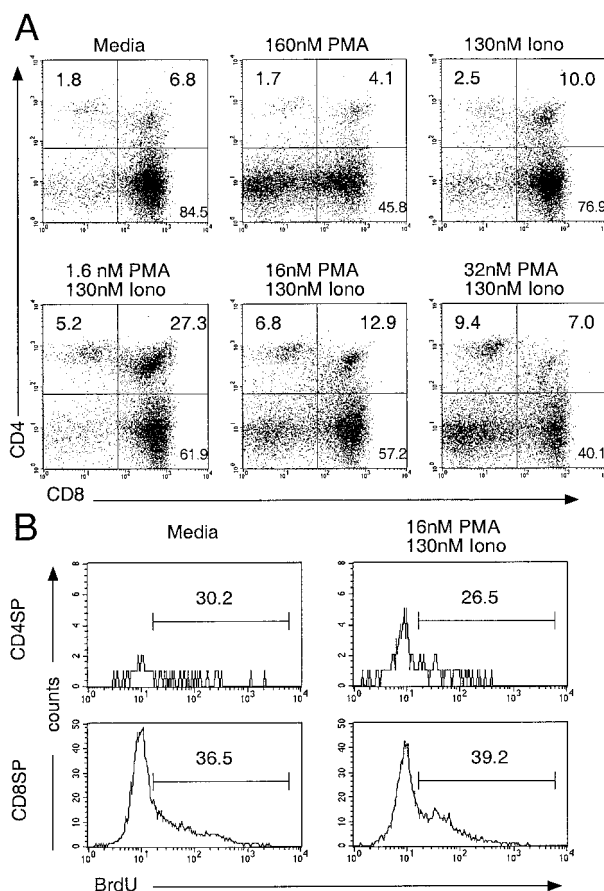


FIGURE 3. PMA and ionomycin can induce the development of CD4SP in MHC class I-specific thymocytes. E16 thymuses from OT-1 mice were cultured for 5 days in the presence of the indicated concentrations of PMA and ionomycin. *A*, Thymocytes were stained with anti-CD4-PE, anti-CD8-Tricolor, and anti-Vα2-FITC. Numbers represent the percentage of live cells within the indicated quadrant. *B*, Thymuses were cultured in the presence of BrdU during the final 16 h before harvest and stained with anti-CD4-PE, anti-CD8-Tricolor, anti-Vα2-biotin + streptavidin-APC and anti-BrdU-FITC. Numbers represent the percentage of BrdU-positive cells within the CD4SP or CD8SP population.

we did not see a more dramatic increase in the absolute number of CD8SPs in C57BL/6 cultures because the population is heterogeneous. These results are consistent with those of Bommhardt et al. (26). In this recent study, PD98059 was shown to enhance CD8 maturation in neonatal thymus organ cultures from either MHC class I- and MHC class II-specific TCR transgenic mice. In addition, they showed that an Ab stimulus that normally gives rise to CD4SPs would promote the appearance of CD8SPs in the presence of PD98059. All of these studies support our original contention that the Erk-signaling pathway is preferentially required for CD4 differentiation, but, in addition, it is now clear that the inhibition of Erk can convert differentiation to the CD8 lineage. Two questions have yet to be addressed: one, can the stimulation of the Erk pathway convert MHC class I-specific cells to the CD4 lineage; and two, how does MHC class II specificity promote Erk activation?

Erk pathway signaling generates CD4SP in class I-restricted thymocytes

To determine whether increased Erk signaling could induce thymocytes that would normally become CD8SP to become CD4SP, we used an FTOC method described by Takahama (27) in which

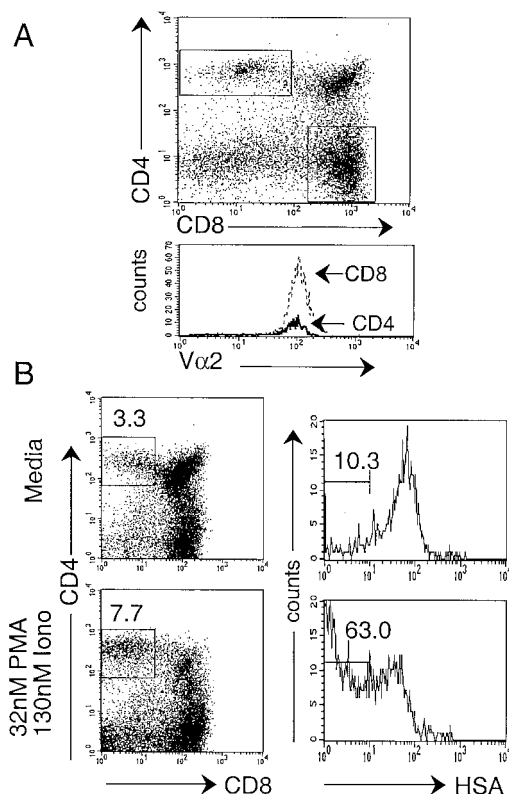


FIGURE 4. MHC class I-restricted CD4SP are mature thymocytes. *A*, The flow cytometric profile represents E16 OT-1 thymuses cultured in the presence of 32 nM PMA + 130 nM ionomycin for 5 days. Thymocytes were triple stained with anti-CD4-PE, anti-CD8-Tricolor, and anti-Vα2-FITC. Histogram represents Vα2 staining of either CD4SP or CD8SP thymocytes as indicated by the dot plot gates. The dotted line represents the Vα2 staining within the CD8SP gate, whereas the thick line represents the Vα2 staining within the CD4SP gate. *B*, E16 OT-1 thymuses were cultured for 5 days in the indicated conditions. Thymocytes were stained with anti-CD4-PE, anti-CD8-Tricolor, anti-Vα2-FITC and anti-HSA-biotin + streptavidin-APC. The profiles represent CD4 and CD8 staining of thymuses. Numbers represent percentage of Vα2⁺CD4SP as indicated by the gate. Histograms represent HSA staining of Vα2⁺CD4SP as indicated.

thymocytes are incubated in the presence of PMA and ionomycin. PMA is known to activate the Erk/MAP kinase pathway through protein kinase C-mediated stimulation of Raf-1 (28). We examined whether PMA-mediated increases in Erk/MAP kinase signaling could alter the fate of MHC class I-restricted OT-1 thymocytes. The OT-1 TCR is formed by the combination of Vα2 and Vβ5 and is specific for the OVA peptide SIINFEKL presented by H-2K^b (22). OT-1 thymuses were cultured for 5 days in the presence of various concentrations of PMA along with 130 nM ionomycin. After culture, thymocytes were analyzed for expression of CD4, CD8, and Vα2. A representative experiment is shown in Fig. 3*A*. Compared with thymuses cultured with vehicle controls or individual reagents, those cultured with a combination of PMA and ionomycin had increased cell number and percentage of CD4SPs that bear high levels of the Vα2 chain. C57BL/6 E16 thymuses cultured with PMA and ionomycin showed no increase in the percentage of CD4SP (data not shown). We also found that addition of PMA increased the DN population. This was noted by Takahama et al. and shown to be an increase in immature cells (27). One possibility is that the increase in CD4SPs is due to proliferation of the preexisting small population caused by PMA and ionomycin. To test this, cultures were set up and labeled with BrdU for 16 h before harvesting. As shown in Fig. 3*B*, the proportion of

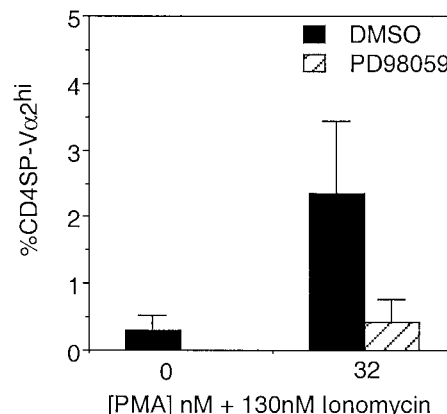


FIGURE 5. The development of MHC Class I-specific CD4SPs is MEK dependent. E16 thymuses from OT-1 TCR transgenic mice were cultured for 5 days in the presence (■) or absence (▨) of 30 μM PD98059 and the presence of the indicated concentration of PMA and ionomycin. Thymocytes were stained for expression of CD4, CD8, and Vα2. Histogram represents the percentage of Vα2⁺CD4⁺ cells with the SD of the mean. The *p* value equals 0.06.

labeled CD4SPs or CD8SPs did not increase with the addition of PMA and ionomycin. This is consistent with results obtained by Takahama (27), and with our observation that PMA plus ionomycin does not increase the proportion of CD4SPs and CD8SPs in cultures from C57BL/6 mice (data not shown).

To further analyze these newly formed CD4SPs for maturity, we compared the level of α-chain expression and the maturation marker heat stable Ag (HSA). As shown, CD4SP induced by PMA plus ionomycin has the same level of Vα2 expression as CD8SP thymocytes (Fig. 4*A*). In some experiments we analyzed the expression of Vβ5 and found, as expected, that it was uniformly high and consistent with exclusion of endogenous receptor rearrangements (data not shown). As such, there is no significant contribution of endogenous TCR chains to the receptor expressed by the CD4SPs, and thus we deduce that development was mediated by an MHC class I-specific TCR. Additionally, PMA plus ionomycin induced a population of Vα2⁺CD4SP cells expressing low levels of HSA that virtually were absent in controls (Fig. 4*B*). This indicates that cells with an MHC class I-specific receptor could be diverted to the CD4SP phenotype by signaling, which included the mobilization of calcium and signaling through the MAP kinase pathway. Finally, to show that the increase in CD4SPs was MEK dependent, thymuses were cultured in the presence of PMA and ionomycin with and without 30 μM PD98059. As shown in Fig. 5, inhibition of MEK using PD98059 inhibits the formation of Vα2⁺CD4SP, suggesting that MHC class I-specific thymocytes can be induced to become CD4SPs in a MEK-dependent manner.

This induction of CD4SP, although small, is consistent with the findings of Takahama et al. (27), in which TCR α-chain-deficient thymuses cultured in the presence of PMA and ionomycin allowed the development of some CD4SP thymocytes. It would appear to us that the CD4SP are converted from the CD8 lineage since both the proportion and number of CD4SPs are reflected in a diminished CD8SP population (data not shown). A possible explanation for the small increase may be that some signals directing cells to the CD8 lineage are dominant over signals to become CD4 (29). As a result, only thymocytes that received the PMA and ionomycin stimulation and did not receive sufficient CD8 lineage-specific signals would be able to become CD4SP. Dominance of 2° fate signals over inductive signals has also been seen in *Caenorhabditis*

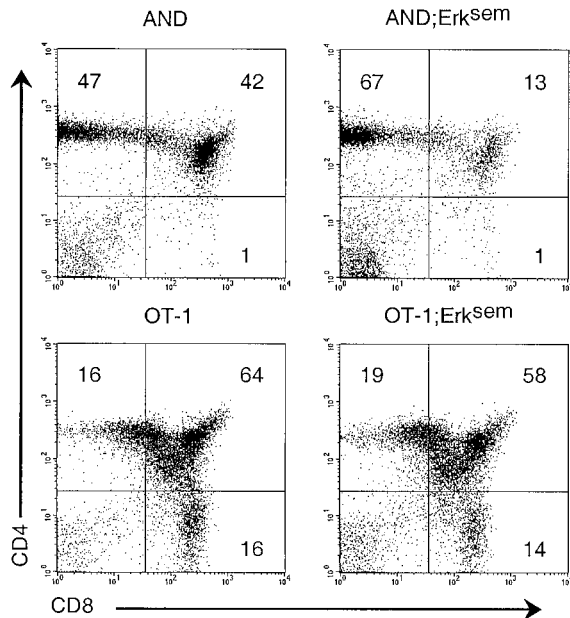


FIGURE 6. Erk2^{sem} does not increase positive selection in MHC class I-restricted mice. Thymocytes were triple stained with anti-CD4-PE, anti-CD8-Tricolor, and anti-V α 2-FITC. Numbers represent the percentage of live thymocytes within the indicated quadrant. This analysis is representative of $n = 4$ mice per group.

elegans vulval development, since overexpression of lin-12 imparts the 2° fate to all vulval precursor cells (30).

Erk2^{sem} does not cause a general increase in positive selection

We have previously shown that a hypersensitive form of Erk-2 (Erk2^{sem}) expressed in transgenic mice causes an increase in the percentage of CD4SPs in normal mice and also in AND, MHC class II-specific TCR transgenic mice (19). To determine whether Erk2^{sem} can promote maturation to the CD8 lineage in TCR transgenic mice, we crossed the Erk2^{sem} mice with the MHC class I-restricted TCR transgenic line OT-1 and compared OT-1 mice with OT-1: Erk2^{sem} mice. For illustration purposes, an analysis of AND and AND: Erk2^{sem} mice is shown. There was no increase in CD8SPs in OT-1: Erk2^{sem} mice whereas, as previously published, there was a substantial increase in the CD4SPs in AND: Erk2^{sem} mice (Fig. 6). Curiously, in the OT-1: Erk2^{sem} mice there was also no consistent increase in the CD4SPs that appear. Given the results presented above, we propose two explanations. One is that the Erk2^{sem} is a hypersensitive, but not a constitutively active, allele and as such does not provide enough activity in this setting to alter the fate of OT-1 T cells. A second possibility is that an increase in Erk activity alone is not sufficient to cause a deviation in lineage commitment. As shown above, PMA alone does not cause the appearance of CD4SPs in OT-1 mice but requires the addition of ionomycin.

The role of p56^{lck} in lineage commitment

The data presented are consistent with a mechanism of lineage commitment that favors differentiation to the CD4 lineage as a consequence of strong signaling mediated through the Erk/MAP kinase pathway. How then does MHC class II recognition lead to a stronger inductive signal? One possibility that has been suggested lies with the MHC-specific coreceptors themselves. CD4 is known to bind to p56^{lck} with a higher stoichiometry than CD8 (31). Thus, signaling through the TCR and CD4 provides for a greater number of activated p56^{lck} molecules when compared with

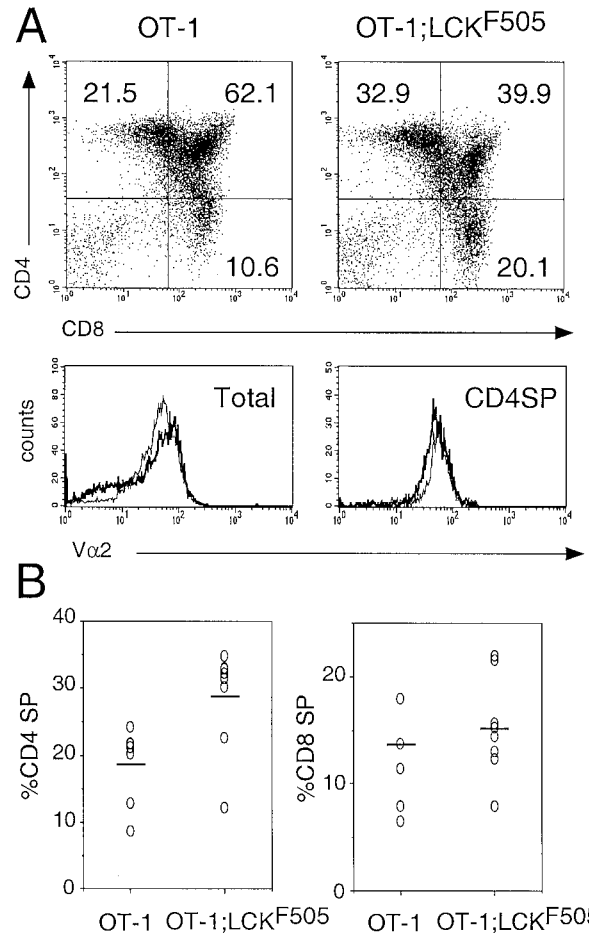


FIGURE 7. Constitutively active p56^{lck}-F505 can induce the development of CD4SP in MHC class I-restricted mice. *A*, Thymocytes were triple stained with anti-CD4-PE, anti-CD8-Tricolor, and anti-V α 2-FITC. Numbers represent the percentage of live cells within the indicated quadrants. Histograms represent V α 2 expression in the total or CD4SP population. The thin line indicates OT-1; the thick line indicates OT-1:p56^{lck}-F505. *B*, Histograms represent the percentage of CD4- or CD8SP cells in OT-1 or OT-1:p56^{lck}-F505 transgenic mice. Circles represent individuals; bar indicates the mean. For CD4SP, $p = 0.007$; for CD8SP, $p = 0.302$ (Student's t test). For OT-1, $n = 7$; for OT-1:p56^{lck}-F505, $n = 8$.

signaling through the TCR and CD8 (11). This, in turn, could lead to a more highly activated MAP kinase cascade. Indeed, the cytoplasmic tail of CD4 and CD8 have both been implicated in lineage commitment (32–35), and the absence of CD4 allows class II-specific T cell precursors to differentiate into CD8SPs (36).

To directly address the role of p56^{lck} in lineage commitment, we introduced an activated form of p56^{lck} (p56^{lck}-F505) into various TCR transgenic mice. As previously published (37), p56^{lck}-F505 caused a potent down-regulation of the TCR in AND TCR transgenic mice (data not shown). However, when combined with the OT-1 TCR transgenes, we found that there was no down-regulation of expression of V α 2 (Fig. 7A) or V β 5 (data not shown), nor were there any changes in cellularity. As an aside, we noticed that OT-1 mice possessed a synthetic α -chain gene driven by an H-2K^k promoter and IgH enhancer (22). Another TCR transgenic line, AD10 (21), using an identical α -chain construct, did not correspond with the results obtained with OT-1; it too showed a strong down-modulation of TCR expression in the presence of p56^{lck}-F505 (data not shown). At this point, we do not understand the fortuitous lack of TCR modulation in OT-1:p56^{lck}-F505 mice.

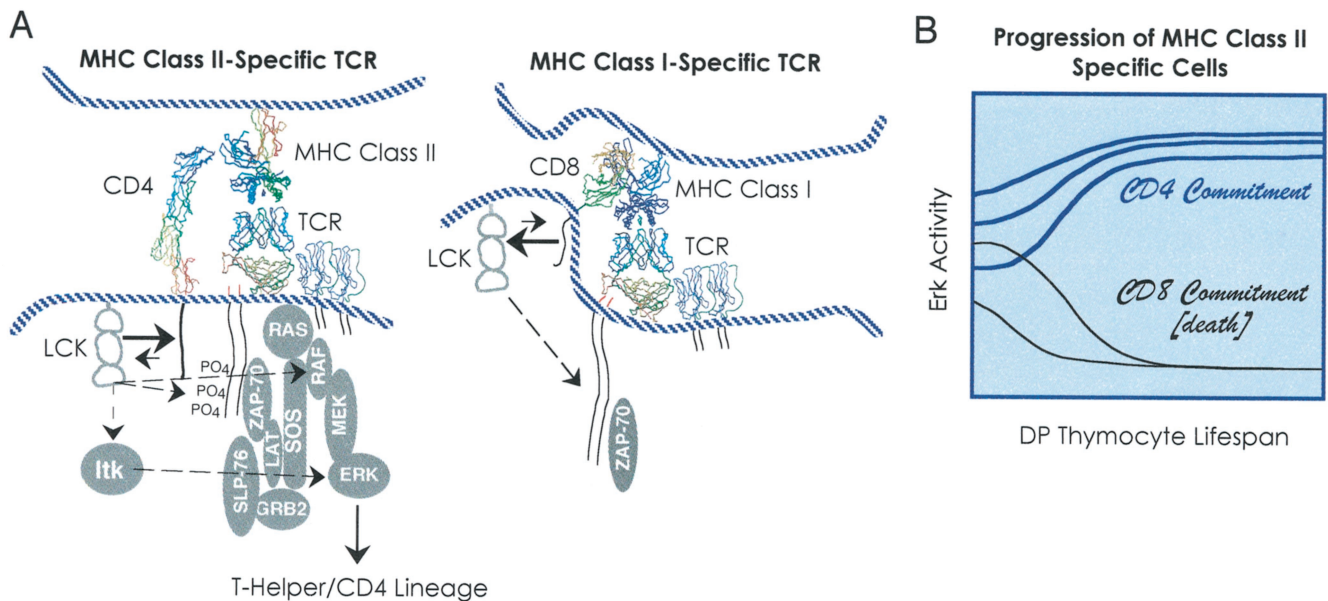


FIGURE 8. Model for $p56^{lck}$ -mediated Erk activation and lineage commitment. **A**, Interaction between TCR, MHC class II, and CD4 allows aggregation and activation of $p56^{lck}$. $p56^{lck}$ mediates high levels of Erk activation directly through interactions with Ras or Raf, or through additional signaling pathways that may include Itk-mediated signaling. High levels of Erk activation promote commitment to the CD4 lineage. Conversely, interactions between the TCR, MHC class I, and CD8 may not engage the same cluster of signaling molecules. Dashed lines represent association and not necessarily phosphorylation events. **B**, A hypothetical progression of Erk signaling as a function of time for MHC class II-specific T cell precursors. Blue lines indicate precursors that have initially made a CD4 lineage commitment, whereas the black lines indicate those that have made a CD8 lineage commitment.

To determine whether $p56^{lck}$ activation can preferentially give rise to CD4SP differentiation, the thymocyte subset proportions were determined for groups of OT-1: $p56^{lck}$ -F505 and OT-1 mice. As shown in Fig. 7B, there was a statistically significant rise in the number of $V\alpha 2^{high}$ CD4SPs but not $V\alpha 2^{high}$ CD8SPs. The percentage of $V\alpha 2^{high}$ CD4SP was increased from 18.7 ± 5.7 in OT-1 mice to 28.7 ± 7.6 in OT-1: $p56^{lck}$ -F505 mice ($p = 0.007$, Student's t test). The percentage of $V\alpha 2^{high}$ CD8SP was not significantly increased ($p = 0.302$, Student's t test). The number of CD4SPs was also increased from an average of 9.03 ± 5.54 million in OT-1 mice to 20.29 ± 13.33 million in OT-1: $p56^{lck}$ -F505 mice. This effect is consistent with the predicted concept of the CD4 and $p56^{lck}$ complex as a mediator of the lineage decision.

Mechanistically, $p56^{lck}$ appears to play a direct role in activating the MAP kinase pathway. Stimulation of $p56^{lck}$ -deficient J.CaM1 cells with anti-CD3 fails to activate Raf-1 and MEK-1 (38). Conversely, thymoblasts containing constitutively active $p56^{lck}$ show constitutive activation Ras, Raf-1, and Erk-1 and -2 (39). Recent reports have shown that the Src homology (SH) 3 domain of Lck is important for activating the Erk pathway and that this Erk activation is independent of CD3 and ZAP-70 phosphorylation (40). This is also consistent with a report showing that $p56^{lck}$ has downstream signaling effects independent of TCR-proximal ZAP-70 activation (41). Furthermore, HIV-induced CD4 aggregation was shown to cause $p56^{lck}$ aggregation and activation, and Raf-1 was shown to be associated with active $p56^{lck}$ in this situation (42). Finally, $p56^{lck}$ was found to be associated with active Erk after stimulation through CD3 and during IL-2-mediated proliferation (43–45).

A basis for lineage commitment

To further examine the role of the Erk/MAP kinase pathway in lineage commitment, we attempted to alter the fate of TCR transgenic thymocytes by varying the levels of Erk/MAP kinase signals. We were able to see an increase in CD4 T cells with specificity for

MHC class I by increasing MAP kinase signaling and, in accord with Bommhardt et al. (26), an increase in MHC class II-specific CD8 T cells by decreasing MAP kinase signaling. A mechanism by which differential signaling can occur is suggested by the increase in CD4SPs promoted by an activated form of $p56^{lck}$. By virtue of its higher stoichiometric association with CD4 vs CD8, MHC class II recognition would result in increased $p56^{lck}$ activation, as compared with MHC class I recognition. In turn, this may result in the activation and possibly the intracellular translocation of Erk-1 and -2 (Fig. 8A). These results could be almost perfectly scripted from basic organogenesis defined for R7 differentiation in the *Drosophila* retina and especially vulva differentiation in *Caenorhabditis*. An inductive pathway originating from a receptor tyrosine kinase, which activates Erk, causes cells to assume a primary phenotype, whereas a weaker inductive stimulus causes cells to assume a secondary phenotype. If we assume CD4 T helper cells to be the primary phenotype, or as Singer et al. described it, the “default pathway,” then T cell lineage commitment fits easily into this now canonical scheme (46). This analogy to development in early metazoans was first suggested by experiments that addressed the role of Notch in T cell lineage commitment (16), and though there is still some controversy concerning the interpretation of these results (17), Notch will almost certainly be involved at some level (47, 48).

As described in the introduction, we propose that the observations to date can be best understood in the framework of an instructive-selective model. No single signaling event may be accurate enough to promote differentiation with the fidelity of the T cell lineage commitment, so that the previously proposed “quantitative model” describes only part of the process. We can imagine that a robust and accurate process would incorporate a degree of fuzziness at any one point in time, but, in a process of serial selection events that continue as T cell differentiate, the accuracy of the lineage selection would be sharpened to a very high degree. This is illustrated by an imaginary progression of MHC class II-specific

thymocyte precursors where the activity of Erk is plotted as a function of the DP lifespan (Fig. 8B). Early in the DP lifespan, high levels of Erk signaling cause an abrupt commitment to the CD4 lineage, and very low levels favor the CD8 lineage. Intermediate levels of Erk signaling may not be decisive, and the direction of differentiation may not be accurately determined with respect to the specificity of the TCR. With time, the commitment process results in the down-regulation of either CD4 or CD8. Those cells that initiated a differentiation to the CD4 lineage may actually increase Erk activation since CD4 and the TCR are up-regulated, whereas those cells that initiated a commitment to the CD8 lineage will experience diminished coreceptor signaling and Erk activation and presumably die. For example, one way of looking at the "rescue" experiments, described above, is that they eliminate the serial proofreading aspect of the lineage decision, since coreceptor expression is constitutive. In a sense, these experiments read out the accuracy of the initiation-signaling bias, and this bias depends on the TCR transgene expressed.

In some examples of lineage commitment, there is a positive feedback aspect that reinforces an initial decision. For example, in the differentiation of proneural equivalence group cells into sensory organ precursors (SOPs) and epidermal cells, nominally equivalent progenitor cells signal one another in a way that is set up to be inherently unstable. Signaling through Notch results in the transcriptional down-regulation of the Delta ligand such that the cell can no longer stimulate Notch activation in its neighbors. The result is a SOP cell expressing the Delta ligand surrounded by epidermal cells expressing Notch. Since the initial expression of Delta cannot be simultaneous in all cells, small variations in expression give rise to islands of SOPs within an equivalence group. In T cell differentiation, we can only speculate that at some point, the thymocyte precursor is bipotent, but begins to acquire a lineage bias with continued TCR-coreceptor signaling. A possibility is that initial Erk signaling causes a block in lineage-specific signaling such as might occur through Notch, or conversely, a lineage-specific signal through Notch may block Erk activation or its downstream effects.

Since, in many examples of signaling, Erk provides a survival signal, a question becomes how do CD8 T cells survive programmed cell death accompanying the absence of positive selection? One possibility is that Erk/MAP kinase signaling is required for survival after the lineage commitment signals have been received. This would explain the reduction of CD8SP as well as CD4SP in dominant negative Ras and dominant negative MEK-bearing mice (49, 50). Another possibility is that Notch is activated in MHC class I-specific thymocytes by an unspecified mechanism. As recently described, Notch provides a potent survival signal to counter dexamethasone-induced apoptosis (17), and thus Notch signaling may provide part of the survival component in the positive selection of CD8 T cells. The increased CD8SPs seen in Notch or Bcl-2 transgenic mice could represent cells that have received a weak TCR-coreceptor signal and would normally have died for a lack of Notch-mediated lineage-specific signal.

If Notch is specifically activated in MHC class I-specific thymocytes, an interesting, but oversimplified, model would be that the mammalian version of suppressor of hairless, CBF, bound to cytoplasmic Notch transcriptionally activates a mammalian enhancer of split, HES, and this acts in concert with corepressors to transcriptionally suppress CD4 transcription (51, 52) through its *cis*-acting silencer (53, 54). A notion is that the Erk/MAP kinase pathway and the Notch pathway somehow form a balance that determines primary vs secondary fates (55); thus far, a molecular mechanism to account for this balance has not been found.

Although we find it interesting to consider the conservation of differentiation mechanisms over a vast evolutionary time frame, we caution that most studies of differentiation in simple metazoans focus on spacial differentiation, and not on cyto differentiation (18). The concept of spacial differentiation is that cells acquire a phenotype based, in part, on their relative position within the developing organism. Arising partly from the circuitry of Notch signaling, as an example, bristle formation is pattern dependent. This is true of most studies, perhaps for the prosaic reason that morphologic differences are easiest to score in a genetic screen. On the other hand, lymphoid development is not an example of spacial differentiation. Although there is lymphoid organ anatomy, cells may not acquire their phenotype on the basis of position. Rather, they differentiate and then migrate to specific areas of primary and secondary lymphoid organs. As such, the signaling circuitry that drives lymphoid differentiation is likely to have many conceptually unique aspects not anticipated by a study of vulva development. The results described in this report, following on the work of many investigators, establishes that there exists a signaling pathway capable of guiding lineage commitment in T cell development. Without question, this is but the smallest glimpse into the intricate mechanisms governing lymphocyte development.

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