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References

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Activation of the Extracellular Signal-Related Kinase/Mitogen-Activated Protein Kinase Pathway Discriminates CD4 Versus CD8 Lineage Commitment in the Thymus

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We have investigated the role of the mitogen-activated protein kinase (MAPK) pathway in the differentiation of CD4+ and CD8+ T cells by looking specifically at the effects of inhibitors of MAPK-activating enzyme, MAPK/extracellular signal-related kinase (ERK) kinase (MEK), during the positive selection step from double-positive to single-positive (SP) thymocytes. Using a variety of transgenic/knockout mouse strain combinations that fail to differentiate individual lineages of SP thymocytes together with genetically engineered F(ab’)2 reagents that induce maturation preferentially to either the CD4 or CD8 subpopulations, we show that induction of CD4 differentiation cells is highly sensitive to levels of MEK inhibition that have no effect on CD8 maturation. In addition, the presence of MEK inhibitor is able to modify signals that normally induce CD4 differentiation to instead promote CD8 differentiation. Finally, we show that continuous culture in the presence of inhibitor interferes with TCR up-regulation in SP thymocytes, suggesting that MAPK signaling may be involved in final maturation steps for both lineages. These data indicate that there is discrimination in the biochemical pathways that are necessary to specify CD4 and CD8 lineage commitment and can reconcile previously conflicting reports on the influence of MAPK activation in commitment and maturation of thymocytes. The Journal of Immunology, 1999, 163: 715–722.

The generation of a useful peripheral T cell repertoire requires the successful completion of selection events that result in T cells whose functional capacity matches their recognition specificity. This is achieved during differentiation in the thymus by a process known as positive selection and minimally involves interactions between the TCR, the coreceptor CD4 or CD8, and the appropriate MHC/peptide ligand.

There has been much interest in the signals that distinguish interactions between MHC class I peptide complexes with TCR/CD8 molecules and MHC class II peptide complexes with TCR/CD4 molecules and lead to commitment to the CD8 and CD4 lineages, respectively. A quantitative signaling model has been suggested (1, 2) in which different strengths of signal are delivered by these respective encounters, based in part on the observation that the coreceptors CD4 and CD8 interact with different efficiencies with the intracellular tyrosine kinase p56Lck (3). Thus, coengagement of TCR plus CD4 with an MHC class II/peptide complex has been suggested to deliver a stronger signal than coengagement of TCR plus CD8 with an MHC class I/peptide complex. An alternative view is that the nature of the signal that specifies differentiation to the CD8 lineage is qualitatively different from that which specifies differentiation to the CD4 lineage. In support of this are observations that receptor engagements that act as antagonists for peripheral T cells, such as TCRs interacting with certain MHC/peptide combinations or CD3 ligation by F(ab’)2, mAb fragments, are particularly good at inducing differentiation to the CD8 lineage for both MHC class I- and class II-restricted TCRs (4–7). In contrast, signals that behave as weak agonists for peripheral T cells are generally efficient at inducing differentiation to the CD4 lineage, again regardless of the primary MHC specificity of the TCR (8–13). Whether these signals specifying lineage commitment differ in degree or substance, at some point there must be a divergence in the signal cascade that ultimately results in the establishment of distinct differentiation outcomes. To date, the point at which the signaling pathways diverge and result in positive selection to the CD4 or CD8 lineage has not been described.

To try and understand the biochemical basis of thymocyte differentiation, we have established an experimental system in which we can mimic CD4 or CD8 lineage commitment signals. We use either bispecific F(ab’)2 Abs (BsAbs), which coligate CD3 with CD4, to induce differentiation to the CD4 subset (13) or monospecific CD3 F(ab’)2 to induce CD8 differentiation (6, 14) in neonatal thymus organ culture (NTOC). Here we examine the contribution of the mitogen-activated protein (MAP) kinase (MAPK) signaling pathway on the induction of CD4 and CD8 thymocyte differentiation. Using the specific MEK inhibitors, PD98059, (15) and U0126 (16), we show that conditions that fully inhibit differentiation of mature CD4 SP cells, whether induced by endogenous ligands or by BsAbs, do not affect differentiation of functional CD8 cells. This is despite the fact that F(ab’)2 reagents that preferentially induce commitment to the CD8 lineage do activate ERK kinases. Furthermore, inhibition of ERK activity modifies a CD4
differsentiation signal to one appropriate for CD8 lineage commitment. Although the presence of the MEK inhibitor does not block commitment to the CD8 lineage, continued presence of the drug blocks TCR up-regulation, suggesting that MEK activity is important for the final stages of maturation for both lineages. Therefore, by identifying discrete requirements for MEK activity in lineage commitment vs maturation our results may resolve some of the conflicting reports regarding the role of MAPK induction in the differentiation of thymocytes. From our data we would suggest that differential activation of the ERK1/2 kinases is an important branch point in the biochemical signals that are required for lineage specification in the thymus.

Materials and Methods

Mice

The βm-deficient (βm<sup>-/-</sup>) and 1-β-deficient (MHC class II<sup>-/-</sup>) mice have been described and were intercrossed to obtain βm<sup>-/-</sup> × class II<sup>-/-</sup> (MHC<sup>-/-</sup>) mice. TCR transgenic mice backcrossed onto a RAG-1-deficient (RAG-1<sup>-/-</sup>) background (19) were as follows: MHC class I-restricted F5 TCR (20), specific for influenza nucleoprotein restricted by H<sup>-2</sup>D<sup>b</sup> and MHC class II-restricted A18 TCR (21), specific for C5 in the context of I-E<sup>e</sup>. Male studs, homozygous for the TCR transgenes on the appropriate backgrounds (RAG-1<sup>-/-</sup>βm<sup>-/-</sup> for F5 and RAG-1<sup>-/-</sup> for A18), were bred with RAG-1<sup>-/-</sup>βm<sup>-/-</sup> or RAG-1<sup>-/-</sup> females, respectively, yielding neonates from which thymus lobes were obtained.

Antibodies

Bi- and monospecific F(ab<sup>'</sup>)<sub>2</sub> Abs dimerized through Fos or Jun leucine zipperers were prepared as described previously (22). Y regions with specificity for CD3ε, CD4, or CD8α were derived from 145.2C11, GK1.5, and YTS169. mAbs were purified and conjugated to FITC or biotin in our own laboratory unless stated otherwise. Rabbit anti-ERK antiserum 122 was a gift from Prof. Chris Marshall (Institute of Cancer Research, London, U.K.).

Thymus organ culture

Neonatal (day of birth) thymus lobes were cultured for 7 days as previously described (6, 13) with the indicated Abs or medium only. MEK inhibitors PD98059 (Calbiochem, La Jolla, CA) or U0126 (gift from Dr. J. M. Trzaskos, DuPont Merck, Wilmington, DE) were added to lobes in culture medium 1–3 h before Ab addition. Unless otherwise stated in the figure legends, inhibitor was replenished either daily or every other day for the first 4 days of culture, after which lobes were transferred to fresh filters in culture medium without Ab or inhibitor for the final 2–3 days of culture to allow re-expression of down-modulated molecules. Single-cell suspensions prepared from lobes after culture were stained with FITC-CD8<sup>e</sup>, PE-CD4 (Boehringer Mannheim, Indianapolis, IN), biotin-HSA (YB5M.10), or biotin-V<sup>b</sup> (KT11.5, for F<sub>5</sub> TCR), -Vβ8.3 (F23.1, for A18 TCR), or pan-TCR-β (H57.597, for βm<sup>-/-</sup>) Abs, followed by streptavidin-Red 670 (Life Technologies, Grand Island, NY), and 20,000 live events (gated on forward and side scatter profiles) were analyzed on a FACSscan (Becton Dickinson, Mountain View, CA).

Proliferation assay

Dendritic cells (DCs) were expanded by culture of C57BL/10 bone marrow in medium supplemented with GM-CSF for 7 days (23). Single-cell suspensions were obtained from equivalent numbers of thymus lobes cultured with 5 µg/ml CD3Fos-F(ab'<sup>'</sup>)<sub>2</sub>, 2 µg/ml CD3/CD4 BsAb, or medium with and without 50 µM MEK inhibitor PD98059 as described above. Thymocytes were stimulated in triplicate wells in 96-well round-bottom tissue culture plates with 2.5 × 10<sup>5</sup> H-2<sup>d</sup>D<sup>b</sup> DCs/well and a titration of NP68 peptide for 5 days at 37°C in medium. Cultures were pulsed with 37 kBq of [3 H]thymidine for 3 days at 37°C in medium. Cultures were pulsed with 37 kBq of [3 H]thymidine/well for the last 18 h of culture, harvested, and counted in a beta counter with scintillation. Data are presented as stimulation index [3 H]thymidine/well for the last 18 h of culture, harvested, and counted in a beta counter with scintillation.

Results

MEK inhibitors block differentiation of CD4 SP and promote CD8 SP thymocyte maturation

The role of the MAP kinase pathway in thymocyte differentiation has been investigated by the expression of both dominant negative ras (24) and mekl (25, 26) constructs in transgenic mice and more recently by using a gain-of-function mutation of erk2, Mekl, and the specific pharmacological inhibitor of MEK, PD98059 (27, 28) with significantly different conclusions. Although these reports agree that activation of the ERK pathway appears unnecessary for negative selection to occur, the dominant negative experiment (25) and one study using the inhibitor PD98059 (28) suggested that ERK activation was required for differentiation from DP to mature SP cells, while another study (27) indicated that inhibition of ERK activity differentially influenced the ratio of CD4:CD8 mature T cells that were produced rather than T cell maturation per se.

Interestingly, it was also shown that introduction of constitutively active forms of MEK (28) or hypersensitive mutants of ERK (27) specifically promoted CD4 SP cell differentiation rather than differentiation of both lineages.

We chose to examine the influence of the MAP kinase pathway in an experimental system we established previously, using in vitro culture of neonatal thymus lobes. Using a variety of transgenic and knockout mouse strain combinations we start with a situation where thymocytes have expanded normally as far as the DP subset and are then completely arrested in their differentiation or during the culture period can differentiate into only a single lineage, unless artificially induced with mono- or bispecific F(ab'<sup>'</sup>)<sub>2</sub> reagents to differentiate to a particular lineage (6, 13, 14). This protocol has enabled us to directly examine the role of the MAP kinase pathway in the commitment decision for differentiation from DP to CD4 or CD8 SP lineages without unduly influencing the transition from DN to DP thymocytes, a stage of differentiation known to require activation of the MAPK pathway (29) and which may be particularly relevant when monitoring differentiation in fetal thymus lobes.

We examined the influence of PD98059 on the differentiation of neonatal thymus lobes from mouse strains that could efficiently differentiate thymocytes into only CD4<sup>+</sup>, CD8<sup>+</sup>, or neither lineage. Thymus lobes that differentiate to the CD8 lineage only were taken from F5/RAG-1<sup>-/-</sup> mice, which have an MHC class I-restricted TCR (20); thymus lobes that differentiate to the CD4 lineage only were obtained from A18A/RAG-1<sup>-/-</sup> mice, which express an MHC class II-restricted TCR (21), or from βm<sup>-/-</sup> mice (17), which lack class I molecules on which to select CD8 SP cells; and finally, thymus lobes from F5/RAG-1<sup>-/-</sup>/βm<sup>-/-</sup> mice were used, which lack both mature T cell subsets.

ERK kinase assay

Thymocytes from MHC<sup>-/-</sup> mice (95% DP, 5% DN cells) were kept at 4°C on ice. Cells were preincubated with 100 µM MEK inhibitor PD98059 for 30 min at 37°C in medium before being stimulated for 7 min with Abs (5 µg/ml). Cells (1 × 10<sup>5</sup> thymocytes/sample) were washed in PBS containing 400 µM Na<sub>3</sub>VO<sub>4</sub>, 5 mM EDTA, and 10 mM NaF; pelleted at 13,000 g and lysed for 1 h at 4°C in 1% Triton X-100, 150 mM NaCl, 50 mM Tris (pH 7.5), 5 mM EDTA, 10 mM NaF, 10 mM disodiumpyrophosphate, 200 mM PMSF, 5 mM leupeptin, 1.5 mM pepstatin A, and 1 mM Na<sub>3</sub>VO<sub>4</sub>. Postnuclear supernatants were precipitated at 4°C with 4 µl of rabbit anti-ERK antiserum and 22 µl of protein A-Sepharose. Precipitates were washed twice in kinase buffer (30 mM Tris (pH 8), 20 mM MgCl<sub>2</sub>, and 2 mM MnCl<sub>2</sub>) and resuspended in 30 µl of kinase buffer plus 10 µM cold ATP and lysed for 1 h at 4°C in 1% Triton X-100, 150 mM NaCl, 50 mM Tris (pH 7.5), and 5 mM EDTA. The reaction was stopped by addition of 2X reducing sample buffer and was resolved on a 12.5% SDS-PAGE gel. Gels were dried and exposed to x-ray film.
Inhibition of MEK has several clear effects on thymus differentiation, as shown by the data represented both as FACS profiles from individual lobes in Fig. 1 and as cell numbers and percentages from pools of lobes in Table I. First, production of mature (HSA low) CD8 \(^{+}\) thymocytes in response to endogenous ligands, e.g., in F5/RAG-1\(^{-}\)labeled lobes is not inhibited and can be enhanced (from 12 to 27\%) by inhibiting MEK during differentiation (Fig. 1A). Moreover, significant enhancement of CD8 SP maturation in

**Figure 1.** MEK inhibitors PD98059 modifies CD4 SP induction signals to promote CD8 SP differentiation. FACS profiles from neonatal thymus lobes are shown after incubation in medium or CD3/CD4 BsAb at 2 \(\mu\)g/ml (A and C) or 2.5 \(\mu\)g/ml (B) in the presence of 50 \(\mu\)M PD98059 where indicated. Inhibitor was added daily from days 0-4 (B) or on days 0, 1, and 3 (A and C), after which the lobes were transferred to fresh medium for the final 3 days of culture. On day 7 lobes were harvested, and single-cell suspensions were counted and triple stained with Abs to CD8, CD4, and HSA. The percentage of cells in the CD4 SP and CD8 SP gates are indicated on the dot plot, with the histogram below showing HSA expression on gated CD8 SP cells. The number on the histogram gives the percentage of cells that are CD8\(^{+}\)HSA low. HSA levels on DP cells are shown for comparison. Similar results were obtained in at least 10 independent experiments.

Table I. Inhibition of MEK promotes CD8 SP cell differentiation and blocks induction of CD4 SP cells

<table>
<thead>
<tr>
<th>Thymus Lobes</th>
<th>PD98059</th>
<th>Medium</th>
<th>CD3/CD4</th>
<th>CD3Fos-F(ab(^{9}))&lt;sub&gt;2&lt;/sub&gt;</th>
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<tr>
<td></td>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>A18A/RAG-1(^{-})</td>
<td>Cell no. (\times 10^{-4})</td>
<td>22.3 ± 5.5</td>
<td>128.0 ± 65</td>
<td>13.0 ± 4</td>
</tr>
<tr>
<td></td>
<td>% CD4 SP</td>
<td>24.9 ± 5.4</td>
<td>1.0 ± 0.16</td>
<td>22.5 ± 13</td>
</tr>
<tr>
<td></td>
<td>% CD8 SP</td>
<td>1.5 ± 0.5</td>
<td>1.8 ± 0.5</td>
<td>4.0 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>Ratio CD4:CD8</td>
<td>17.0</td>
<td>0.56</td>
<td>5.6</td>
</tr>
<tr>
<td>F5/RAG-1(^{-})/β2m(^{-})</td>
<td>Cell no. (\times 10^{-4})</td>
<td>16.7 ± 9.1</td>
<td>56.0 ± 7.8</td>
<td>14.3 ± 4.9</td>
</tr>
<tr>
<td></td>
<td>% CD4 SP</td>
<td>2.0 ± 0.9</td>
<td>0.02 ± 0.01</td>
<td>16.3 ± 5.0</td>
</tr>
<tr>
<td></td>
<td>% CD8 SP</td>
<td>2.5 ± 1.3</td>
<td>11.0 ± 1.3</td>
<td>4.2 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>Ratio CD4:CD8</td>
<td>0.08</td>
<td>0.002</td>
<td>3.9</td>
</tr>
</tbody>
</table>

* Neonatal thymus lobes were cultured for 4 days with 5 \(\mu\)g/ml CD3/CD4, CD3Fos-F(ab\(^{9}\))<sub>2</sub>, or medium with daily addition of 50 \(\mu\)M PD98059. Lobes were transferred to fresh medium without Abs or inhibitor for an additional 3 days before FACS analysis. SP subsets were gated on mature HSA low cells, and data were obtained from means of triplicate lobes ± SD.
the presence of PD98059 was observed even in the absence of class I MHC ligands (F5/RAG-1<sup>m<sub>es</sub></sup> mice, see Table I). The presence of bovine β2m in the culture medium probably promotes stabilization of a few MHC class I molecules as some mature CD8<sup>+</sup> cells can be recovered from these cultures; the numbers are significantly reduced when cultured with medium supplemented with β2m<sup>es</sup> mouse serum M. A. B and R. Z., (M.A.B. and R.Z., unpublished observation). Inclusion of MEK inhibitor was able to significantly increase the percentage of mature CD8<sup>+</sup> cells that differentiate from 2.5% to 11% (Table I, F5/RAG-1<sup>m<sub>es</sub></sup>/β2m<sup>es</sup> ).

Second, differentiation of CD4 SP cells on endogenous ligands is dramatically inhibited in the presence of MEK inhibitor, both for class II-restricted TCR transgenic cells (A18A/RAG-1<sup>m<sub>es</sub></sup>; Fig. 1B and Table I), and for cells expressing polyclonal TCRs from β2m<sup>es</sup> mice (Fig. 1C). Indeed, inhibition of MEK is even able to block CD4 SP thymocyte differentiation induced by artificial stimuli such as CD3/CD4 BsAb (Fig. 1A, F5/RAG-1<sup>m<sub>es</sub></sup> mice, and Table I, F5/RAG-1<sup>m<sub>es</sub></sup>/β2m<sup>es</sup> mice). Moreover, in addition to inhibiting differentiation of CD4<sup>+</sup> cells, the effect of MEK inhibition on lobes cultured with CD3/CD4 BsAb is to change a stimulus that normally promotes efficient CD4<sup>+</sup> differentiation to one that induces differentiation of CD8<sup>+</sup> cells (Fig. 1, A and B, and Table I). Finally, induction of CD8<sup>+</sup> cell differentiation by culture of lobes with CD3Fos-F(ab′)<sub>2</sub>, which we have shown to be an efficient inducer of CD8 maturation (6), is frequently enhanced in the presence of PD98059 (Table I), confirming that inhibition of MEK does not inhibit but, rather, promotes differentiation to the CD8<sup>+</sup> lineage.

We confirmed that the effect on thymocyte maturation was a direct consequence of inhibiting MEK kinase activity by using a second recently described, noncompetitive inhibitor of MEK, UO126 (16), in cultures of thymus lobes from F5/RAG-1<sup>m<sub>es</sub></sup>/β2m<sup>es</sup> mice. As illustrated in Fig. 2, UO126 inhibition of MEK has the same consequence as inhibition by PD98059, even though the former blocks MEK kinase activity on downstream targets, while the latter blocks MEK activation by Raf. UO126 shows more pronounced inhibition of CD4<sup>+</sup> cells than PD98059, consistent with its ability to block constitutively active MEK, and yet still enhances differentiation of CD8<sup>+</sup> cells.

In all cases the maturity of the CD8<sup>+</sup> population that developed was assessed by staining with Abs to HSA and TCR as markers for maturation. In our early experiments MEK inhibitor was added continuously throughout the 7 days of lobe culture at a concentration (25–50 μM) that inhibited the response of mature T cells to Ag by about 80% (data not shown). Using this protocol we found that PD98059 gave very good inhibition of CD4<sup>+</sup> cells and enhanced production of CD8<sup>+</sup> cells; however, the CD8<sup>+</sup> cells that developed, while having down-regulated the maturation marker HSA, did not up-regulate TCR to normal mature levels. Subsequently, we modified our protocol and only added the MEK inhibitor for the first 3–4 days of culture during the time the F(ab′)<sub>2</sub> reagents were present and washed out both Ab and inhibitor for the final culture period (this latter protocol was used for the data presented in Figs. 1 and 2 and Table I). A direct comparison on TCR up-regulation of F5/RAG-1<sup>m<sub>es</sub></sup>/β2m<sup>es</sup> lobes cultured in PD98059 continuously or for the first 3 days only is shown in Fig. 3. Whereas removal of the inhibitor has little influence on the degree to which CD4<sup>+</sup> differentiation is inhibited or CD8<sup>+</sup> differentiation is enhanced, there was a clear inhibition of TCR up-regulation on mature CD8<sup>+</sup> cells if the inhibitor was present throughout culture. This observation suggests that whereas MEK kinase activity may not be required for CD8 lineage commitment, it is required for some of the final maturation stages that result in the characteristic phenotype of mature CD8 SP cells. These data may also explain the failure to observe the generation of CD8 SP thymocytes in the presence of dominant negative ras (24) and mekl (25, 26) constructs in transgenic mice or in the presence of PD98059 in studies where TCR up-regulation is used to define maturity (28).

Over a number of experiments it was found that incubation with the MEK inhibitor showed no toxicity on the thymus lobes at concentrations ≤50 μM; indeed, cell yields were frequently increased in cultures that had been incubated with PD98059 (Table I). We considered whether the increase in CD8 SP cells arose as a result of PD98059 protecting the DP cells from apoptosis during culture, particularly in the presence of the CD3/CD4 BsAb, which we have previously found to cause apoptosis of some DP thymocytes (13). However, when lobes are cultured with another compound that previously found to cause apoptosis of some DP thymocytes (13).

### Table I

<table>
<thead>
<tr>
<th>Culture with:</th>
<th>% Mature CD4&lt;sup&gt;+&lt;/sup&gt; TCR&lt;sub&gt;high&lt;/sub&gt; Thymocytes</th>
<th>% Mature CD8&lt;sup&gt;+&lt;/sup&gt; TCR&lt;sub&gt;high&lt;/sub&gt; Thymocytes</th>
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<tr>
<td>Medium</td>
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<td><img src="#" alt="CD3/CD4" /></td>
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<tr>
<td>CD3fos-F(ab′)&lt;sub&gt;2&lt;/sub&gt;</td>
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</tr>
<tr>
<td>+ PD98059</td>
<td><img src="#" alt="+ PD98059" /></td>
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</tr>
<tr>
<td>+ UO126</td>
<td><img src="#" alt="+ UO126" /></td>
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</table>

**FIGURE 2.** A noncompetitive MEK inhibitor, UO126, also inhibits CD4<sup>+</sup> differentiation and promotes CD8<sup>+</sup> differentiation. Neonatal thymus lobes from F5/RAG-1<sup>m<sub>es</sub></sup>/β2m<sup>es</sup> mice were cultured for 4 days with 5 μg/ml CD3Fos-F(ab′)<sub>2</sub> or 2 μg/ml CD3/CD4 or medium, with addition of 30 μM PD98059 or 9 μM UO126 on days 0, 1, and 3. Lobes were transferred to fresh medium on day 4 without Abs or inhibitors for an additional 3 days before FACS analysis. SP subsets were gated on mature TCR<sub>high</sub> cells.

Mature CD8 SP cells that differentiate in the presence of MEK inhibitor are functional

Given that we could show that continuous culture in MEK inhibitor prevented full T cell maturation and resulted in CD8<sup>+</sup> T cells...
that had low levels of TCR, it was important to ascertain that the cells that differentiated in cultures from which inhibitor had been removed for the final few days were functional.

Cells were harvested from NTOCs from either F5/RAG-1<sup>−/−</sup>/B<sub>2</sub>m<sup>−/−</sup> mice, which differentiated in medium with and without PD98059, or from F5/RAG-1<sup>−/−</sup>/B<sub>2</sub>m<sup>−/−</sup> mice, which were also induced to differentiate with CD3/CD4 and CD3Fos-F(ab′)<sub>2</sub> reagents in the presence or the absence of PD98059, and were stimulated with APCs plus antigenic peptide, NP68. Proliferation was measured on day 3 of culture, and the specific stimulation index obtained in the presence of peptide compared with that in medium controls is shown in Fig. 4A. In all cases CD8 cells that differentiated in the presence of PD98059 responded to peptide. These data confirm that CD8<sup>+</sup> cells that mature in the presence of MEK inhibitors are capable of responding to Ag.

**Stimulation of thymocytes with either CD3Fos-F(ab′)<sub>2</sub> or CD3/CD4 activates ERK kinase activity**

CD3 ligation in the absence of costimulation has been shown to act as an anergic signal for T cell clones (31). Furthermore, the biochemical signals resulting from ligation of CD3 by F(ab′)<sub>2</sub> Abs on

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**FIGURE 3.** Sustained inhibition of MEK prevents up-regulation of TCR in CD8 SP thymocytes. Neonatal thymus lobes from F5/RAG-1<sup>−/−</sup>/B<sub>2</sub>m<sup>−/−</sup> mice were cultured in medium, 5 μg/ml CD3/CD4 BsAb, or 5 μg/ml CD3Fos-F(ab′)<sub>2</sub> as indicated, from days 0–3 of culture. Lobes labeled PD98059 continuous had 25 μM PD98059 added on days 0, 1, 2, 3, and 6, while those labeled PD98059 removed d3 had 50 μM PD98059 added on days 0, 1, and 2 only. The percentages of CD4 and CD8 SP cells that arose in the cultures are indicated on the dot plots, and the levels of TCR on gated DP (filled histogram) and CD8 SP cells (black line) are indicated in the histograms below. Note that the presence of the MEK inhibitor inhibits CD4 differentiation in response to CD3/CD4 BsAb and increases the percentage of CD8<sup>+</sup> cells that arise in medium or in the presence of Ab; however, continuous presence of the inhibitor prevents TCR up-regulation in CD8<sup>+</sup> cells.
T cell clones (32) or mature thymocytes (6) have been shown to resemble those induced by antagonist peptides. It has been reported that T cell clones exposed to antagonistic peptides fail to activate ERK kinases, in contrast to clones exposed to antigenic peptides (33–35). Given that inhibition of MEK activity did not interfere with induction of CD8 differentiation by CD3Fos-F(ab’\(^9\))\(_2\), we investigated whether ligation of receptors on DP thymocytes with CD3Fos-F(ab’\(^9\))\(_2\) or CD3/CD4 BsAb would induce activation of the ERK kinases.

Thymocytes from MHC\(^{\text{neg}}\) mice were used in the assay for ERK activity to exclude any contribution from mature SP thymocytes. Thymocyte suspensions were pretreated for 30 min in medium alone or in medium containing MEK inhibitor and then stimulated with Abs at 37°C for 7 min. As shown in Fig. 5, activation of ERK kinase activity, as measured by phosphorylation of MBP, is stimulated by incubation of thymocytes with intact 2C11 Ab, CD3Fos-F(ab’\(^9\))\(_2\) or CD3/CD4 BsAb, and in all cases this activity is reduced to background levels by preincubation of cells with the inhibitor PD98059. There was some variability in the extent to which the different stimuli induced ERK kinase activity, and in the experiment shown in Fig. 5, CD3Fos-F(ab’\(^9\))\(_2\) gave stronger activation of ERK than CD3/CD4 BsAb. However, over five independent experiments, in which the extent of ERK activation after stimulation varied between 2- to 5-fold above the background level, there was no clear distinction in the ability of one stimulus to induce higher levels of ERK kinase activity than the other. These data agree with a recent report that showed that, despite its ability to antagonize T cell responses, CD3Fos-F(ab’\(^9\))\(_2\) induced similar levels of ERK activation to that induced by CD3/CD4 BsAb in T cell clones at early time points (36). Importantly, the data clearly indicate that at these concentrations of MEK inhibitor the kinase activity induced by these stimuli is reduced to background levels. Therefore, it appears that despite the observation that ERK kinase activity is induced by stimuli that promote both CD4 and CD8 maturation, this activity is critical for differentiation of CD4, but not CD8, SP thymocytes.

**FIGURE 4.** CD8 SP cells, which differentiate in the presence of MEK inhibitor, respond to Ag. Neonatal thymus lobes were culture with medium, 2 \(\mu\)g/ml CD3/CD4, or 5 \(\mu\)g/ml CD3Fos-F(ab’\(^9\))\(_2\) in the presence of 50 \(\mu\)M PD98059 for 4 days before transfer to fresh medium. On day 7 single-cell suspensions from equivalent numbers of lobes were resuspended to the same volume and stimulated with B10 dendritic cells with or without NP68 peptide Ag. A. Wells were harvested on day 3 after pulsing with 18.5 kBq/well \([\text{\textsuperscript{3}}\text{H}]\)thymidine for the last 18 h of culture. Data are presented as the stimulation index in response to 100 nM peptide divided by spontaneous proliferation in medium alone. B. In a separate experiment, cells were sampled from the cultures after 24 h and stained for expression of CD69, CD8, CD4, and TCR. Data are presented as the percentage of CD8\(^+\) cells expressing CD69 (black area) among the total percentage of CD8\(^+\) cells in cultures of medium alone or stimulated with 10 nM peptide.

**FIGURE 5.** Stimulation of ERK kinase activity in thymocytes by ligation of CD3 is inhibited by PD98059. Phosphorylation of myelin basic protein by anti-ERK immunoprecipitates is shown after thymocytes from MHC\(^{\text{neg}}\) mice were stimulated with 1) medium alone; 2) anti-CD3 Ab (2C11); 3) CD3Fos-F(ab’\(^9\))\(_2\); and 4) CD3/CD4 BsAb for 7 min. Where indicated, cells were preincubated with 100 \(\mu\)M PD98059 for 30 min before stimulation with Abs, which was shown to reduce the ERK kinase activity to background levels.
Discussion

The biochemical basis of the signals that specify lineage commitment in the thymus are not well understood. To try to decipher these events we have established a system in which we can specifically induce differentiation of DP thymocytes to the CD4 or CD8 lineages using Ab F(ab')₂ fragments to mimic TCR coreceptor engagements (6, 13, 14). Here we show that differentiation of the CD4 lineage is acutely sensitive to inhibition of the MAPK pathway, whereas CD8 lineage determination is not. Indeed, our data show that by inhibiting the MEK signaling pathway, we can alter a signal that normally results in CD4 maturation to one that favors CD8 maturation.

These data are in agreement with the studies of Sharp et al. (27), who showed with a gain-of-function ERK transgene that activation of ERK promoted differentiation of CD4 SP cells. In addition, they showed that culture of day 17 thymus lobes with the same MEK inhibitor used in this study, PD98059, produced a reversal in the CD4:CD8 SP ratio resulting from a reduction in the number of CD4 cells. The latter suggested that MEK activity was important for positive selection of CD4, but not CD8, cells. In the current study we use neonatal rather than fetal thymus lobes and can confirm that when starting with a thymus containing differentiated DP cells, two independently acting MEK inhibitors, PD98059 and UO126, specifically block selection of DP thymocytes to the CD4 lineage. CD4 differentiation was significantly arrested by the inhibitor whether the selecting ligand was endogenous MHC:peptide complex or whether we used CD3/CD4 BsAbs to induce maturation.

In contrast to CD4 differentiation, CD8 cell maturation was generally enhanced in the presence of the MEK inhibitor both to endogenous ligand and to stimulation with either CD3Fos-F(ab')₂ or with CD3/CD4 BsAb. Furthermore, the CD8 SP cells that arose not only had a mature phenotype (HSA<sub>low</sub> TCR<sub>high</sub>)<sup>th</sup>, but were fully responsive to Ag. The differential sensitivity of the CD4 vs the CD8 lineage to the effects of the inhibitors suggests that the primary influence is on the thymocytes themselves, particularly when differentiation is induced by F(ab')₂ reagents that only bind thymocytes. However, we cannot rule out the possibility that the MEK inhibitors influence the expression of genes in thymic stromal cells, such as Notch or its ligands (37), which may have additional influence on differentiation of CD8 cells.

It is unlikely that the levels of MEK inhibitor we added to the thymus lobes were sufficient to completely block MEK activity during the first few days of culture, and therefore, it is possible that low levels of ERK activation are also required for development of mature CD8 SP cells. Involvement of the Ras → MEK → ERK signal transduction pathway in thymocyte-positive selection has been suggested from the experiments of Alberola-Ila et al. (25, 26), who showed that dominant negative forms of Ras or MEK, both individually and particularly in combination, could completely inhibit positive selection of both SP lineages. However, we also showed that activation of the ERK pathway is involved in processes other than the lineage commitment signal. For example, we found that although daily addition of the MEK inhibitor did not impede production of HSA<sub>low</sub> CD8 SP cells, these cells had not fully up-regulated TCR levels to those normally found in mature T cells. In contrast, replenishing the MEK inhibitor daily while the inducing Abs were present during the first few days of culture, but removing both Ab and inhibitor for the last 3 days of culture, allowed differentiation of TCR<sub>high</sub> CD8 cells and yet still specifically blocked CD4 SP differentiation. These data suggest that high levels of ERK activity may not be required for the CD8 lineage commitment decision, but may be essential for other aspects of maturation such as up-regulation of the TCR, and the continuous presence of a DN form of the enzyme or the pharmacological inhibitor (28) would not permit these later maturation stages to occur.

From our earlier studies we observed that the signals that favored CD4 SP cell differentiation, namely limited coligation of CD3 with the coreceptors, behave as weak agonists for peripheral T cells, whereas the signals that favor CD8 commitment behave as T cell antagonists (6). If this is generally true, it is perhaps not surprising that activation of the ERK kinase pathway is required for CD4 differentiation but not for CD8 differentiation, as a number of groups have shown that stimulation of mature T cells with antagonist peptides does not lead to activation of Ras/ERK kinases, unlike activation with agonist ligands (33–35). In view of this, we were surprised to observe that treatment of thymocytes with CD3Fos-F(ab')₂ was able to efficiently stimulate ERK kinase activity, as CD3Fos-F(ab')₂ behaves as a powerful antagonist for peripheral T cells and T cell clones (6, 32). However, a recent report (36) has shown that although stimulation of a T cell clone with either CD3Fos-F(ab')₂ or CD3/CD4 BsAb resulted in similar levels of ERK-1 and ERK-2 activity at early time points; the ERK activity after CD3Fos-F(ab')₂ treatment decayed more rapidly, similar to what we previously showed for ZAP-70 activation in thymocytes in response to these stimuli (6).

We have proposed a model of lineage commitment that suggests that the discriminatory signals that direct differentiation to either the CD4 or CD8 subsets are a function of the extent to which Src family kinases, particularly Lck, are activated at the same time as the TCR is engaging ligand in the thymus (6, 14). Thus, engagements of the TCR that stimulate significant activation of Lck are most appropriate for directing cells to the CD4 SP lineage, whereas limited activation of Lck results in commitment to CD8. The data presented here would suggest that one consequence of stimulating Lck and a requirement for commitment to the CD4 lineage would be a progression of signals through the Ras → MEK → ERK pathway. In contrast, although artificial stimuli that result in CD8 differentiation may also activate the ERK kinases, this activation and presumably the immediate downstream consequences on gene transcription are not required for the CD8 commitment decision.

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