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The Role of Erk1 and Erk2 in Multiple Stages of T Cell Development

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Summary

Activation of extracellular-signal-regulated protein kinase (Erk) is central to growth-factor-receptor-mediated signaling including that originating from the T cell antigen receptor. It integrates cytoplasmic signals to effect changes in transcription associated with differentiation, proliferation, and survival. In this report, we present an analysis of mice with targeted deletions in *Erk1* and *Erk2* to assess the relationship between Erk activity and cell-cycle progression, thymocyte development, and lineage commitment. These studies show that Erk is selectively retained during β selection-driven proliferation, and yet Erk1/2 are not required to complete differentiation to $CD4^+CD8^+$ preselection stage of development. Erk activity is essential for the process of positive selection, and it differentially affects CD4 and CD8 T cell maturation; yet, diminished expression itself is not sufficient to alter lineage commitment.

Introduction

T cell development proceeds in an ordered progression that includes at least three important checkpoints. The first major checkpoint occurs at an early DN3 ($CD4^-CD8^-CD44^-CD25^+$) stage in development, a stage that depends upon signals from the pre-TCR (T cell antigen receptor). Pre-Tα associates with the product of the TCR β chain gene to convey the presence of a productive TCR β chain gene rearrangement. Signaling from pre-TCR requires such molecules as Lck and Lat (Molina et al., 1992; Zhang et al., 1999), and this sets off a number of differentiation events that include: downregulation of CD25 and RAG; six to eight rounds of cell division; and differentiation to the $CD4^+CD8^+$ (DP) stage of development. The second checkpoint, termed positive selection, passes DP thymocytes for promotion to CD4 or CD8 T cells. It is based on TCR specificities for self-encoded peptides associated with MHC class II or MHC class I molecules expressed on the cortical epithelium. Positive selection confers upon the T cell population an ability to receive persistent survival signals through the TCR and interrogate antigen-presenting cells throughout the body for the presence of self and foreign peptides. Positive selection also involves many of the same TCR-linked signaling components important for pre-TCR signaling, although the outcome is very different. Signals through the TCR are intimately involved in the fate decision to differentiate into either a CD4 helper T cell or a CD8 cytotoxic T cell, but they do not promote cell division. A third checkpoint follows positive selection, and it is based on the TCR recognition of self-peptide-MHC complexes present on dendritic cells and medullary epithelial cells (Kappler et al., 1987; Guidos et al., 1990; Egerton et al., 1990; Anderson et al., 2002). At this stage, the outcome of TCR signaling is once again quite different, and it results in cell death. The signaling cascades of negative selection are poorly characterized but appear to culminate in the activity of Bim (Villunger et al., 2004).

A number of studies have implicated the MAP kinase pathway in the program of T cell development. Dominant-interfering and constitutively active forms of Mek1 revealed that the MAP kinase pathway is important for the first β selection checkpoint (Crompton et al., 1996), and studies using Mek1 inhibitors were consistent with this result (Sharp et al., 1997; Bommhardt et al., 1999). The transgenic expression of dominant-interfering and constitutively active forms of Ras, Raf, or Mek1 all implicated the MAP kinase pathway in positive selection (Swan et al., 1995; Alberola-Ila et al., 1995; O'Shea et al., 1996), though none of these studies suggested a role for this pathway in the CD4 versus CD8 lineage-commitment decision. In contrast, the expression of a gain-of-function (hypersensitive) *Erk2* transgene or the use of Mek1 inhibitors in FTOC showed that the strength of signal through the MAP kinase pathway can differentially promote the development of CD4 or CD8 T cells (Sharp et al., 1997; Bommhardt et al., 1999; Sharp and Hedrick, 1999). Further work showed that duration of the MAP-kinase-pathway signals can influence lineage commitment (Kaye, 2000; Adachi and Iwata, 2002; Nishida et al., 2004).

Erk1/2 constitute a focal point of the MAP-kinase-pathway signaling in mammalian cells. These two highly homologous serine-threonine kinases are activated by tyrosine and threonine dual phosphorylation and, in turn, broadcast this activation to both cytoplasmic signaling complexes and nuclear transcription factors (Chang and Karin, 2001; Pouyssegur et al., 2002; Morrison and Davis, 2003; Alberola-Ila and Hernandez-Hoyos, 2003). Erk is likely to be the origin of a ramifying signal-transduction program that affects many aspects of cell physiology. Mice deficient in signaling intermediates upstream of Erk1/2 exhibit a severe defect in positive selection (Priatel et al., 2002; Wen et al., 2004), whereas mice deficient for transcription factors known to be downstream of Erk1/2 exhibit somewhat less se-

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vere phenotypes (Costello et al., 2004; Bettini et al., 2002; Rivera et al., 2000). The implication is that there is redundancy or that other pathways may emanate from Erk1/2 and affect the outcome of TCR-mediated signaling. The role of the MAP kinase pathway in negative selection is less well characterized, although most studies have not found that it makes an important contribution (Swan et al., 1995; Alberola-Ila et al., 1996; Sharp et al., 1997; Bommhardt et al., 2000; Mariathasan et al., 2000).

In order to determine the importance of Erk1 and Erk2, both together and individually, we have analyzed mice selectively deficient for *Erk1* (*Mapk3*), *Erk2* (*Mapk1*), or both *Erk1* and *Erk2*. Previous results showed that *Erk1*-deficient mice are viable and ostensibly normal (Pages et al., 1999), whereas *Erk2*-deficient mice exhibit early embryonic lethality (Hatano et al., 2003; Yao et al., 2003; Saba-El-Leil et al., 2003) (C.D.K. and S.M.H., unpublished results). To overcome early lethality, we generated mice in which the *Erk2* gene was conditionally deleted at two different stages in thymic development. The analyses of these mice reveal that the MAP kinase pathway differentially affects at least two stages in thymic development.

Results

Mice with a Hemizygous *Erk2* Deficiency Exhibit Embryonic Lethality

Germline deletion of *Erk2* was accomplished with a targeting vector as depicted in Figure S1 in the Supplemental Data available with this article online. From 60 $Erk2^{+/-}$ × $Erk2^{+/-}$ offspring, no homozygous mice were born. In addition, $Erk2^{+/-}$ mice were backcrossed to C57BL/6 in two separate experiments, and the number of heterozygous $Erk2^{+/-}$ progeny was tallied. As shown in Figure S2, the number of progeny with a hemizygous *Erk2* deficiency decreased such that at generation four, only one $Erk2^{+/-}$ mouse was recovered. When a generation-three mouse was crossed to strain SVJ/129, the *Erk2*-deficient allele transmitted at a frequency of 50%. There would seem to be a strict requirement for the full level of Erk2 expression during development that can be dominantly suppressed by one or more genes present in the SVJ/129 strain. We did not determine the stage at which development was aborted in the hemizygous *Erk2*-deficient embryos, although we note that three other *Erk2* mutant strains exhibited homozygous lethality at three different embryonic stages (Saba-El-Leil et al., 2003; Yao et al., 2003; Hatano et al., 2003).

To avoid embryonic lethality, we modified the *Erk2* locus by homologous recombination for conditional deletion (Figure 1A). Female mice containing the targeted allele ($Erk2^{fn}$) were bred with *Ella-cre* transgenic mice, and the progeny were screened for a recombination event that retained exon 3 but deleted PGK-neo ($Erk2^{f}$) (Lakso et al., 1996; Holzenberger et al., 2000). Mouse strains with either the $Erk2^{f}$ or $Erk2^{fn}$ alleles were used to assess the requirement for *Erk2* in T cell development.

Deletion of *Erk2* in DN3 Thymocytes Decreases Thymic Cellularity

Germline transmission of each allele was tested by Southern blotting and PCR. Homozygous $Erk2^{f/f}$ and $Erk2^{fn/fn}$ mice were found to express the same levels of Erk2 protein and have normal T cell development indicating that the modification of the *Erk2* locus with or without PGK-neo did not disrupt gene regulation (Figure 1B). Both alleles were crossed with *pLck-cre* transgenic mice in order to express the cre recombinase in the T cell lineage prior to the β selection checkpoint (Lee et al., 2001; Wolfer et al., 2002). The two alleles were comparably expressed and deleted with no detectable differences (Figure 1B). In order to determine the stage of *Erk2* deletion, we sorted DN3 and DN4 populations and tested them for exon 3 deletion and Erk2-protein loss (Figure 1C). At the DN3 stage, exon 3 was still detectable by PCR amplification, and there remained a substantial amount of protein. At the DN4 stage, we detected virtually no remaining Erk2 protein.

In mice deleted for Erk2, the numbers of total and DP thymocytes were reduced by about 30%, consistent with a partial block in the expansion associated with the DN-to-DP transition or a reduction in cell survival (Figure 1D). In order to examine this directly, we analyzed the DN subpopulations, as measured by the expression of CD25 and CD44, were analyzed (Figure 1E). Indeed, there was a substantial increase in the number of DN3 thymocytes, consistent with a partial block in the DN3 to DN4 maturation step.

Deletion of Both Erk1 and Erk2 Does Not Prevent Maturation to the DP Stage of Development

To determine the roles of Erk1/2 in the DN-to-DP transition, we crossed $Erk2^{fn/fn}$;*pLck-cre* mice to mice with a germline deficiency in *Erk1* (Pages et al., 1999). As described above, thymocytes from $Erk2^{f/f}$;*pLck-cre* mice showed a partial block at the DN3 stage (Figures 1E and 2A), although cells progressed to the DN4 stage and beyond. Densitometry of Erk2 protein (Figure 1C) revealed that its expression was decreased approximately 3-fold at the DN3 stage and from 25–100-fold at the DN4 stage. More importantly, in $Erk1^{-/-}$;$Erk2^{f/f}$;*Lck-cre* mice, the thymocytes progressed to the DN4 stage and, as we will show below, were competent to progress to the DP stage of development with no detectable Erk1/2 protein remaining.

To investigate this partial block, we looked at two major characteristics of DN-thymocyte development—proliferation and TCR β chain expression. The rate of thymocyte proliferation in vivo was assessed by injecting bromodeoxyuridine (BrdU) and harvesting the thymocytes 3 hr later. The result was that thymocytes from $Erk2^{fn/fn}$;*pLck-cre* mice exhibited normal or even slightly enhanced BrdU uptake in the DN4 population (Figure 2B). Surprisingly, this was true for thymocytes from $Erk1^{-/-}$;$Erk2^{fn/fn}$;*pLck-cre* mice as well. In addition, we found a high degree of variability in the mice deficient for both *Erk* isoforms. Whereas some mice had very small thymuses (13 × 10^6 cells) and few DN4 cells, others had larger thymuses (115 × 10^6 cells) with only a moderate block at the DN3 stage of development (Figure 2C). To determine the effectiveness of Erk2 deletion in the absence of *Erk1*, we isolated the DN3 and DN4 populations by cell sorting and analyzed them for Erk2 by western blotting. The results showed that, in the absence of *Erk1*, Erk2 protein was unchanged at the DN3 stage and was reduced but still detectable at the DN4

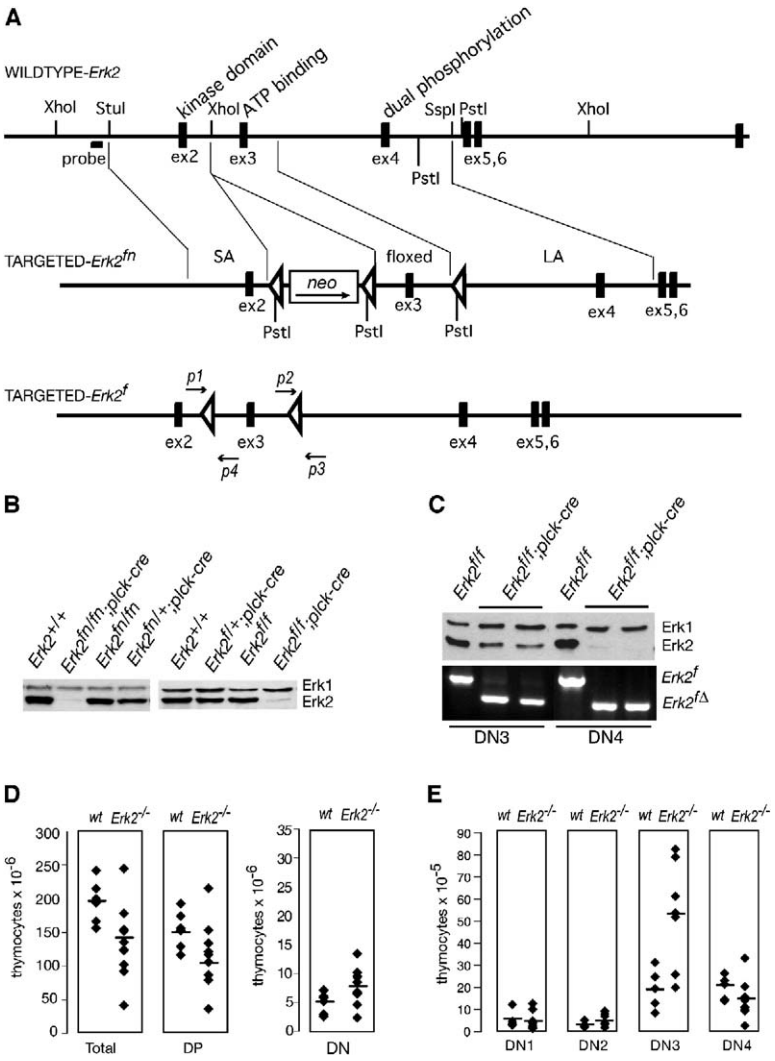

Figure 1. Loss of *Erk2* in Early Thymocyte Development Causes a Decrease in Thymic Cellularity

(A) A diagram representing the *Erk2* locus and targeting strategy used to generate two targeted versions of the *Erk2* allele.

(B) Western-blot analysis of Erk protein in total thymocyte lysates from mice with indicated genotypes.

(C) Western-blot analysis and *Erk2* exon 3 deletion in sorted DN3 ($CD4^-CD8^-B220^-H\text{-}2A^{b-}\gamma\delta\ TCR^-CD44^-CD25^+$) and DN4 ($CD4^-CD8^-B220^-H\text{-}2A^{b-}\gamma\delta\ TCR^-CD44^-CD25^-$) thymocytes from $Erk2^{f/f}$ and $Erk2^{f/f}$;*pLck-cre* mice. Genomic *Erk2* exon 3 deletion PCR was carried out with primers p1, p2, and p3 as depicted in Figure 1A (see Experimental Procedures for sequences).

(D) Graphical representation of total thymocyte numbers and DP thymocyte numbers. Data represent six $Erk2^{fn/fn}$ (*wt*) mice and nine $Erk2^{fn/fn}$;*pLck-cre* ($Erk2^{-/-}$) mice.

(E) Graphical representation of DN subset numbers. Subsets are classified as DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$), and DN4 ($CD44^-CD25^-$). Data represent six $Erk2^{fn/fn}$ (*wt*) mice and nine $Erk2^{fn/fn}$;*pLck-cre* ($Erk2^{-/-}$) mice.

stage (Figure 2D). These data are consistent with the selective survival of DN3 and DN4 cells that retain Erk2.

The decrease in cell number (Figure 2C) could also be associated with increased cell death, although flow cytometric analyses of directly explanted cells did not reveal an excess of apoptotic cells either by staining with annexin V or by forward and side light scatter (data not shown). Because apoptotic cells are rapidly engulfed, they may be detected only by an in situ analysis. Nonetheless, all of the mice deficient for *Erk1* and *Erk2* possessed a substantial population of DP thymocytes with no detectable protein expression remaining (see below).

A second parameter we tested was the expression of the TCR β chain. *Erk*-deficient DN3 cells expressed normal levels of intracellular β chain, indicating that rearrangement and protein production were intact (Figure 2E). At the DN4 stage, there was an increased population of cells from *Erk2*-deficient and *Erk*-deficient mice that expressed no intracellular β chain. This result was found consistently for *Erk2*-deficient DN4 thymocytes defined as $B220^-MHCII^-TCR\gamma\delta^-CD4^-8^-25^-44^-90^+$ cells (data not shown). These cells thus exhibit characteristics of DN4 cells without β chain expression. Although γδ T cells were gated out in this analysis, we considered the possibility that these cells could represent an increased population of γδ T cells. Subsequent analyses showed no significant change in the percentage of γδ T cells within the DN subset (data not shown).

These experiments reveal that that Erk is required for proliferation and differentiation associated with β selection, such that in the absence of Erk1, there is a selection for the retention of a small amount of Erk2. We infer that under physiological conditions, Erk expression is in excess over that which is required for continued proliferation. As an aside, we note that the *pLck-cre* transgene itself affects T cell development to an extent (Mertsching et al., 2002), and it is thus essential in carrying out experiments of this type to compare *cre* transgenic mice with and without *loxP*-flanked alleles.

The Expression of Erk1/2 Is Essential for Positive Selection

Thymocyte populations were measured by the expression of the CD4 and CD8 coreceptors, TCR β chain, and CD69. As expected, the thymocyte subsets from

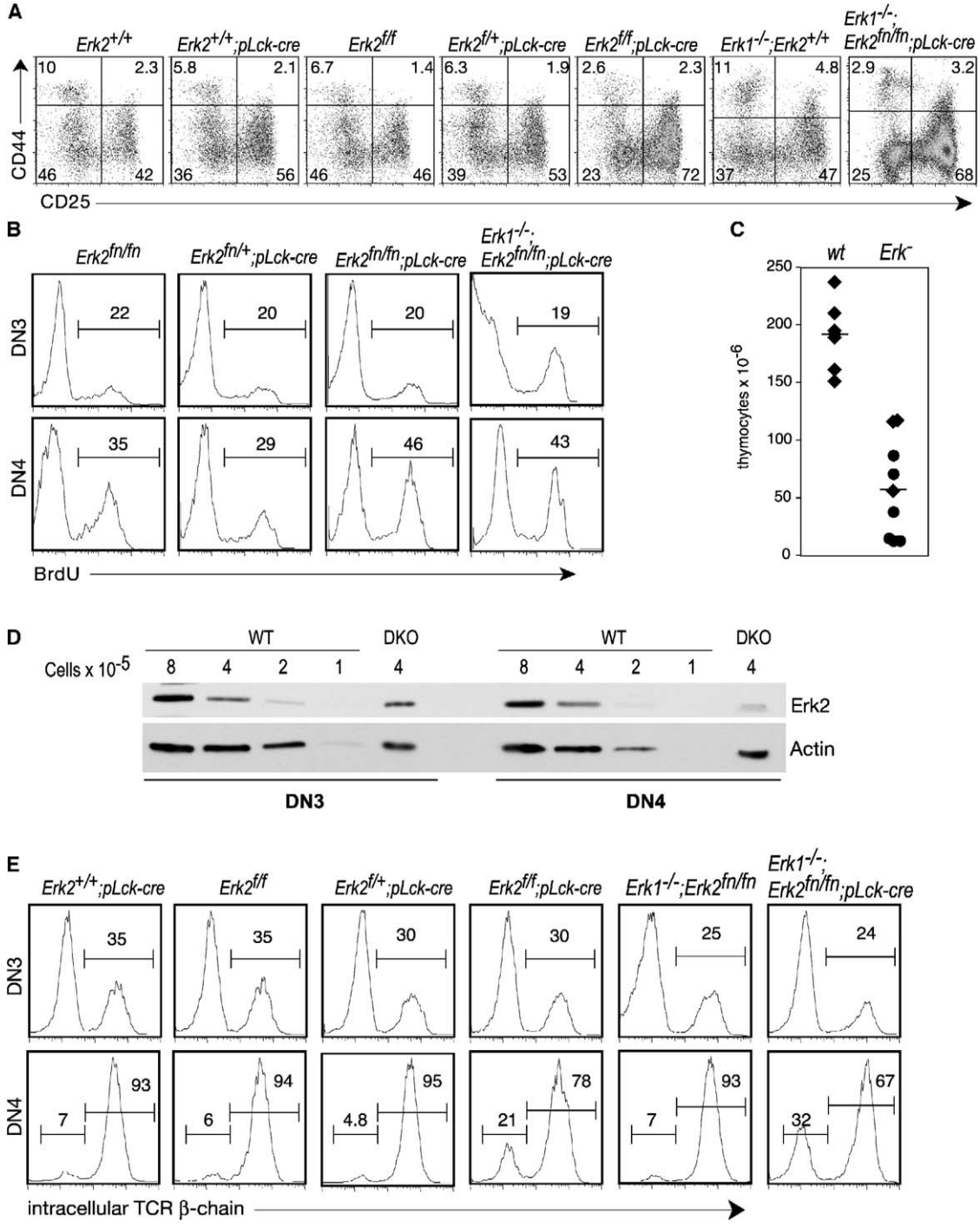

Figure 2. *Erk2*-Deficient Thymocytes Have a Partial DN3 Block

(A) DN thymocytes ($CD4^-CD8^-B220^-H\text{-}2A^{b-}\gamma\delta TCR^-$) from the indicated mice were stained for CD44 and CD25. Population percentages are indicated in the quadrants.

(B) DN3 and DN4 thymocytes from mice with indicated genotypes were injected and stained to indicate BrdU incorporation.

(C) Histogram of total numbers from nine $Erk2^{fn/fn}$ (*wt*) mice and nine $Erk1^{-/-};Erk2^{f/f};pLck\text{-}cre$ (*Erk*$^-$) mice; ♦ = male and ● = female.

(D) Western-blot analysis of Erk expression from sorted DN3 and DN4 thymocytes from $Erk2^{fn/fn}$ (*wt*) and $Erk1^{-/-};Erk2^{fn/fn};Lck\text{-}cre$ (DKO) mice.

(E) DN3 and DN4 thymocytes from above mice were stained for intracellular β chain expression.

$Erk2^{f/f}$, $Erk2^{fn/fn}$, and wild-type mice were not significantly different (Figure 3A), although the introduction of *pLck-cre* transgene appears to have caused a small decrease in mature thymocytes. The phenotype of $Erk1^{+/+};Erk2^{f/+};pLck\text{-}cre$ mice was also similar to *pLck-cre* mice, and this is consistent with our analysis of thy-

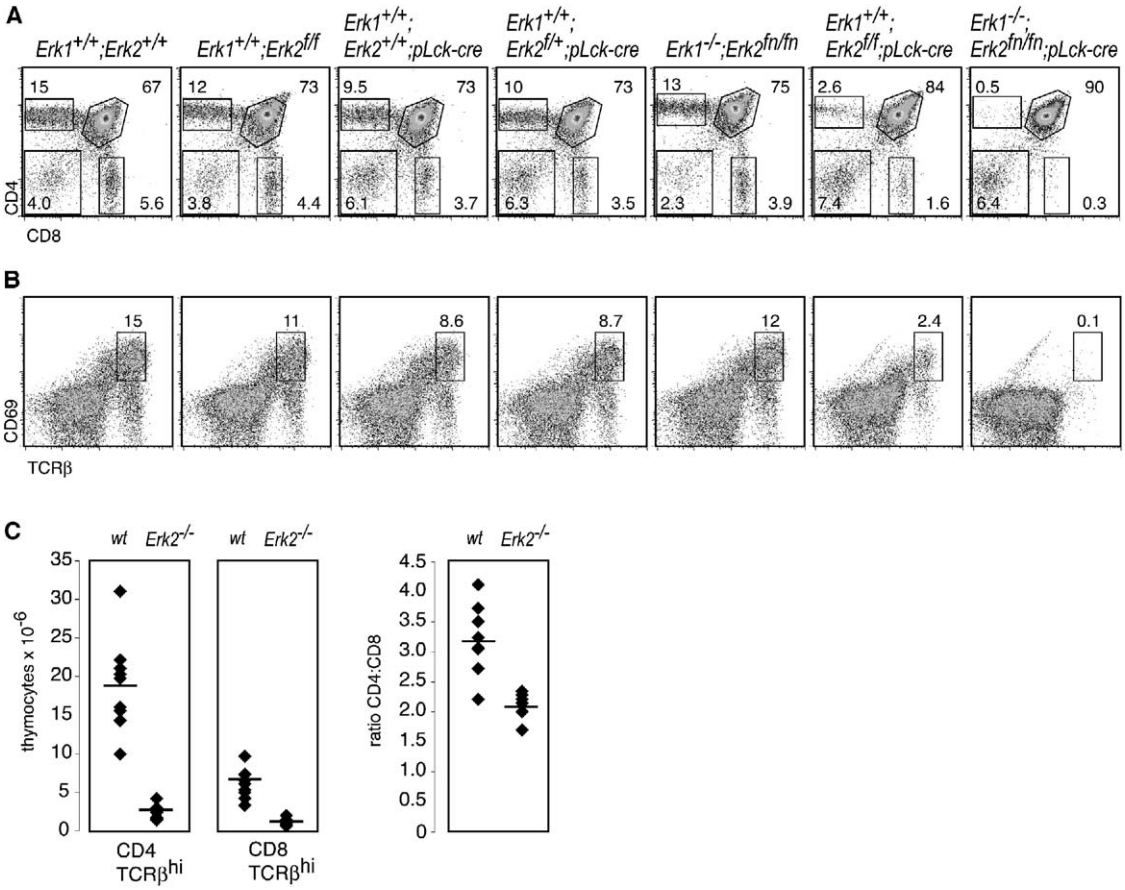

Figure 3. Erk Activity Is Essential for Positive Selection

(A) CD4 and CD8 flow cytometric analyses of thymocyte populations were carried out from the indicated mice.

(B) Staining of total thymocytes from above mice with antibodies against CD69 and TCR.

(C) Mature (TCR^{hi}) CD4 and CD8 thymocyte numbers from nine *Erk2*$^{fn/fn}$ (*wt*) mice and six *Erk2*$^{fn/fn}$*;plckcre* (*Erk2*$^{-/-}$) mice. CD4/CD8 ratio is a calculation of the number of TCR^{hi} CD4 divided by the number of TCR^{hi} CD8.

mocytes from *Erk2*$^{+/-}$ mice (Figure 3A and data not shown). In this analysis, we also did not find a difference in the thymus subsets from mice that were deficient only for *Erk1*. We surmise that the difference between this and the previous report may be ascribed to genetic variations caused by backcrossing to C57BL/6 mice (Pages et al., 1999). In contrast, loss of both Erk isoforms in *Erk1*$^{-/-}$*;Erk2*$^{fn/fn}$*;pLck-cre* mice was permissive for the appearance of a substantial DP population, but it was not permissive for the positive-selection-associated appearance of mature CD4 or CD8 T cells.

Because the levels of TCR β chain and CD69 increase with positive selection, we used a bivariant plot of these two cell-surface molecules to reveal the percentage of cells undergoing positive selection. As shown, a small number of thymocytes undergo positive selection in the presence of Erk1, whereas virtually no mature cells were detected in the absence of both isoforms (Figure 3B). These results show that the development of a small number of single positive thymocytes in *Erk2*$^{f/f}$*;pLck-cre* mice was dependent on the activity of Erk1, and this verifies previous results showing that Erk1 plays a role in positive selection (Pages et al., 1999). We can conclude that quantitatively, Erk2 is substantially more important than Erk1, although we cannot say whether this is due to abundance or activity.

Because Erk1 and Erk2 are both expressed in thymocytes and appear to be at least partially redundant, a deficiency in *Erk2* alone constitutes the equivalent of a hypomorphic mutation in this essential signaling pathway. With this in mind, we plotted the number of CD4 and CD8 thymocytes found in *Erk2*-deficient mice and their wild-type littermates. As depicted in Figure 3C, although there is a decrease in both the CD4 and CD8 subsets, CD4 thymocytes are preferentially affected such that the CD4:CD8 ratio goes from 3.2 in wild-type mice to 2.2 in *Erk2*-deficient mice. An implication of this result is, minimally, that CD4 T cell differentiation exhibits a greater dependence on Erk signaling compared with CD8 differentiation.

Erk2$^{fn/fn}$*;Cd4-cre* Mice Have Defects in Positive Selection

Because *Erk2*$^{f/f}$*;pLck-cre* mice demonstrated a partial block in DN3 to DN4 progression, we were concerned that this might carry over to later stages of development. Therefore, to address the role of Erk2 in DP thymocytes, we bred mice with the *Erk2*^{fn} allele to coex-

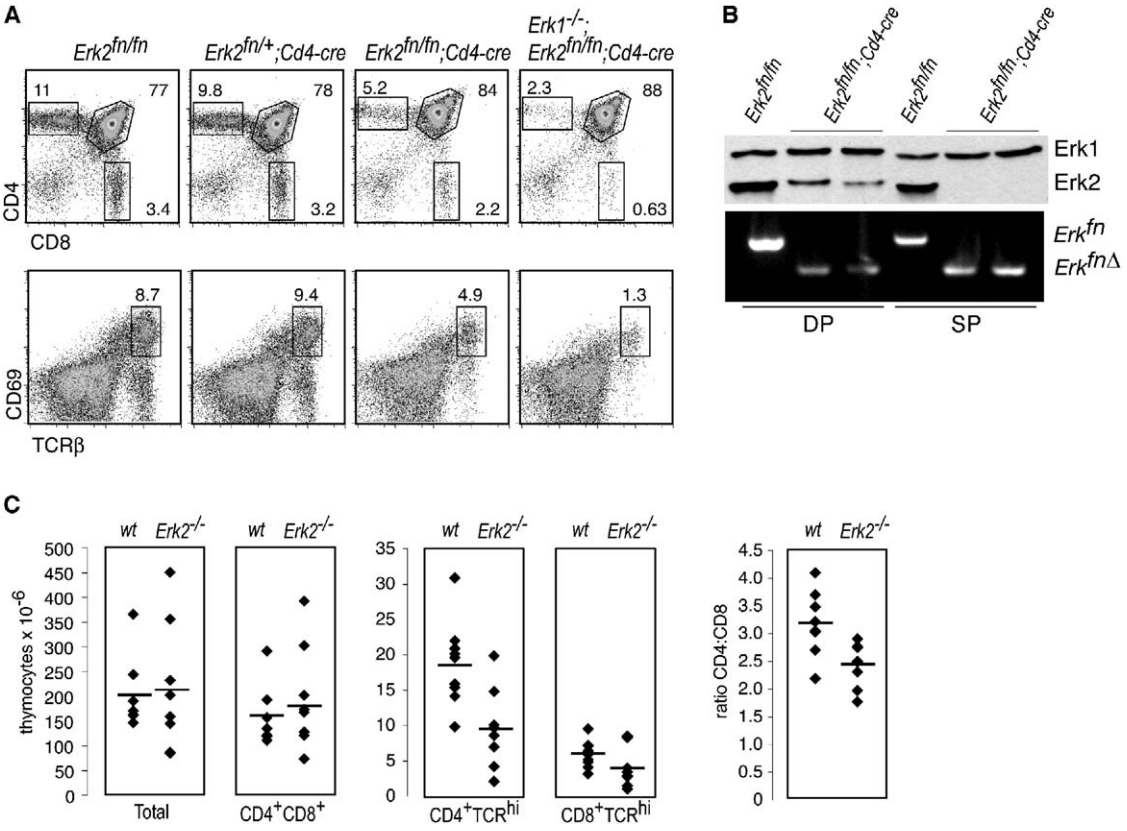

Figure 4. *Erk2*$^{fn/fn}$*;CD4cre* Mice Exhibit Normal Cellularity and Defective Positive Selection

(A) CD4 and CD8 flow cytometric analyses of thymocyte populations were carried out from the indicated mice. The top row depicts staining with antibodies specific for CD4 and CD8, and the bottom row depicts staining with antibodies specific for CD69 and CD3.

(B) Western-blot analysis and *Erk2* exon 3 deletion in sorted DP (CD4$^+$CD8$^+$) and SP (CD4 single positive) thymocytes. PCR was performed as in Figure 1.

(C) Histogram representation of total and subpopulation thymocyte numbers and CD4/CD8 ratio. Data from nine mice *Erk2*$^{fn/fn}$ (*wt*) mice and eight (*Erk2*$^{-/-}$) *Erk2*$^{fn/fn}$*;Cd4-cre* mice were graphed.

press the later-deleting *Cd4-cre* transgene. Figure 4A depicts the thymocyte subset analysis from a representative set of mice with different *Erk* genotypes. Once again, mice with a hemizygous *Erk2* deficiency were within the range expected for wild-type mice, whereas *Erk2*$^{fn/fn}$*;Cd4-cre* mice exhibited a decrease in the number of CD4 and CD8 SPs. This phenotype was accentuated by a deficiency in *Erk1*. Thymocytes that had recently undergone positive selection were again enumerated by measuring the relative expression levels of CD69 and the TCR β chain. By this measure, there was a significant reduction in the percentage of positively selected thymocytes in both *Erk2*$^{fn/fn}$*;Cd4-cre* and *Erk1*$^{-/-}$*;Erk2*$^{fn/fn}$*;Cd4-cre* mice. In comparison to deletion with the *pLck-cre* transgene, overall thymic cellularity was restored in *Erk2*$^{fn/fn}$*;Cd4-cre* mice with an increase in the proportion of DP thymocytes and a compensatory decrease in single positive thymocytes (Figure 4C). In contrast to *Erk1/2*-deficient mice generated with the *pLck-cre* transgene, *Erk1*$^{-/-}$*;Erk2*$^{fn/fn}$*;Cd4-cre* mice retained significant populations of SP thymocytes.

In order to determine the efficiency of deletion with the *Cd4-cre* transgene, we sorted DP and CD4 thymocytes and tested them for Erk2 protein and the *Erk2* exon 3 deletion. Although DP thymocytes had completely deleted exon 3 of *Erk2* as measured by PCR, residual Erk2 protein was easily detectable. The turnover of Erk2 is such that it did not persist in CD4SP thymocytes (Figure 4B). Again, a decrease in the amount of Erk expressed was correlated with a decrease in the CD4-to-CD8 ratio of mature TCR^{hi} thymocytes (Figure 4C). We conclude that the few mature thymocytes produced in *Erk1*$^{-/-}$*;Erk2*$^{fn/fn}$*;Cd4-cre* mice most likely achieved maturity as a result of enduring Erk2 protein in the DPs.

Erk Deficiency Preferentially Affects the CD4 Lineage

To further investigate the effects of decreased Erk2 activity on lineage commitment, we crossed the *Erk2*$^{fn/fn}$*;Cd4-cre* or *Erk2*$^{fn/fn}$*;pLck-cre* mice with TCR transgenic mice: either an MHC-class-II-specific TCR (*AND*) (Kaye et al., 1989) or an MHC-class-I-specific TCR (*OT-I*) (Hogquist et al., 1994). For comparison, we analyzed TCR transgenic mice with wild-type *Erk1/2* loci or a germline *Erk1* deletion.

The presence of an Erk1 deficiency did not result in an alternation in the thymocyte subsets, and this is consistent with our analysis of T cell development in

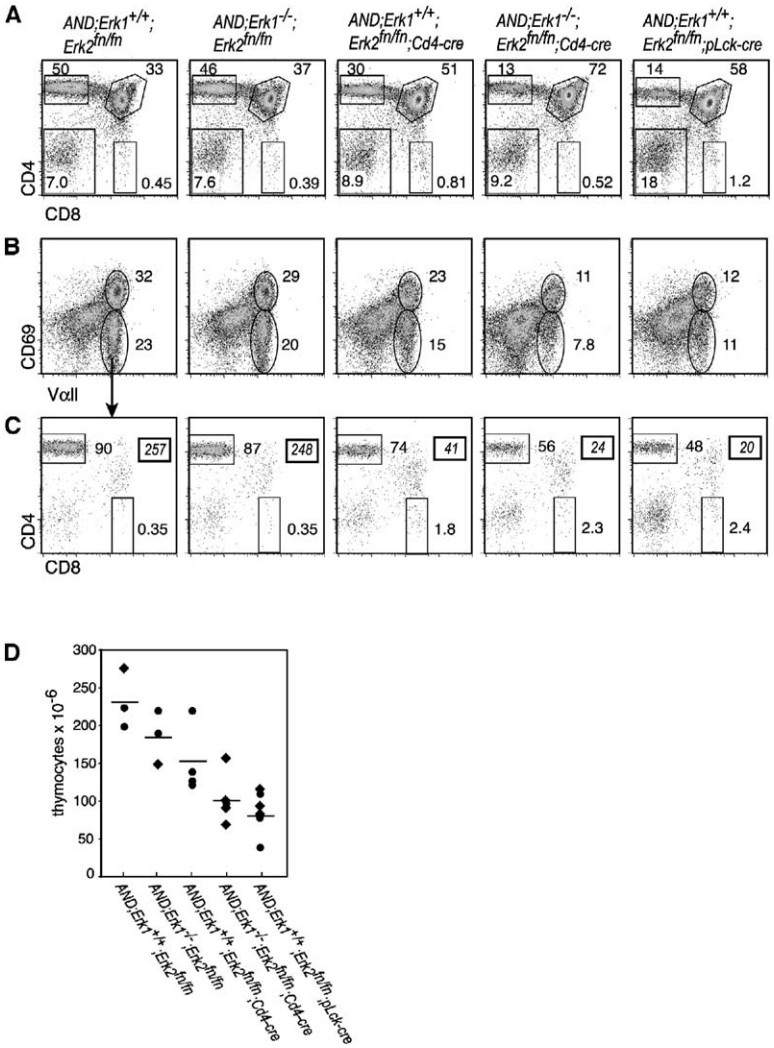

Figure 5. Erk2 May Not Determine Lineage Commitment but Is Important for Progression of the CD4 Lineage

(A) Staining of total thymocytes from the indicated strains with antibodies specific for CD4 and CD8.

(B) Staining with antibodies specific for CD69 and Vα11.

(C) The CD69$^-$Vα11$^+$ (mature) cells were analyzed for the expression of CD4 and CD8. The percentages of CD4$^+$ and CD8$^+$ cells that fall within that gate are given. The boxed numbers represent a ratio of percentage of mature CD4 and CD8 T cells.

(D) Histogram of total thymocytes from the indicated mice.

Erk1-deficient mice without a TCR transgene. Although there are apparent subtle differences in the examples presented in Figure 5, these differences were within the range of values characteristically found for *AND* mice (Figure 5A and 5B). In contrast, *AND;Erk2$^{fn/fn}$;Cd4-cre* mice exhibited an altered proportion of DP and CD4 populations (Figure 5A). This was accompanied by a reduction in the percentage of TCRhi, CD69hi, and CD69lo thymocytes in *AND;Erk2$^{fn/fn}$;Cd4cre* mice, suggesting inefficient positive selection. Positive selection was diminished further in *AND;Erk1$^{+/+}$;Erk2$^{fn/fn}$;pLck-cre* mice and in *AND;Erk1$^{-/-}$;Erk2$^{fn/fn}$;Cd4-cre* mice, reflecting the reduced levels of total Erk molecules in these two genotypes.

To reveal the percentage of cells that have completed positive selection, we gated the CD69^{lo}Vα11^{hi} cells for CD4 and CD8 (Figure 5C). This analysis revealed that the number of CD4 cells diminished with levels of expressed Erk, and these cells were not replaced by mature CD8 thymocytes. As such, a simple decrease in Erk activity is not sufficient to cause MHC-class-II-specific thymocytes to differentiate into the CD8 lineage. We did notice that the ratio of CD4 to CD8 T cells changed dramatically. Whereas AND mice exhibited a CD4-to-CD8 ratio of 257 in this example, *AND;Erk2$^{fn/fn}$;pLck-cre* mice exhibited a CD4-to-CD8 ratio of 20 (Figure 5C, insert box). This was a consistent trend in dozens of mice of different genotypes, and we interpret it to mean that CD8 development has less of a requirement for Erk-dependent signal transduction. Because the cells were gated for a Vα11^{hi} phenotype, we consider the possibility that these cells express a second TCR α chain to be unlikely. The total number of thymocytes was also found to reflect the amount of Erk (Figure 5D), even though the *Cd4-cre* transgene becomes transcriptionally active subsequent to the expansion associated with β selection. Because there is minimal proliferation in the later stages of thymic development, this decrease in cell number could reflect diminished cell survival or accelerated migration into the peripheral lymphoid organs.

A different result was obtained in the analysis of *OT-I* mice. The development of CD8 thymocytes was not as sensitive to the deletion of the *Erk2* gene (Figures 6A–6C). Previous work has shown that at least a subset of CD4$^+$CD8^{lo} thymocytes have received a posi-

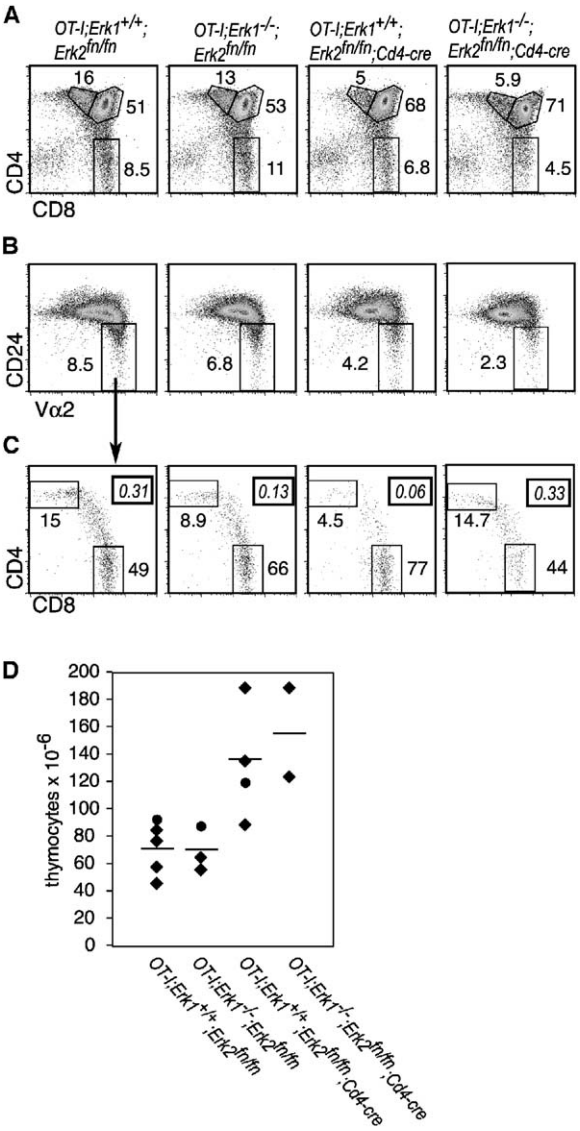

Figure 6. Maturation of CD8 T Cells in *OT-I* Mice Is Insensitive to Erk Expression

(A) Total thymocytes were stained with antibodies specific for CD4 and CD8.

(B) Thymocytes were stained with antibodies specific for CD24 (heat-stable antigen, HSA) and Vα2. Mature T cells were defined as $CD24^{lo}V\alpha2^{hi}$.

(C) Mature T cells were analyzed for the expression of CD4 and CD8.

(D) The total numbers of thymocytes from the indicated strains were plotted.

tive-selection signal and may or may not be committed to one of the two T cell lineages (Lucas and Germain, 1996; Suzuki et al., 1997). Consistent with this idea, in thymuses from *OT-I* mice decreases in Erk correlated with decreases in the percentage of $CD4^+CD8^{lo}$ thymocytes, and this decrease was not entirely compensated by the increase in thymocyte cell number (Figures 6A and 6D). Yet, the maturation of CD8 *OT-I* thymocytes was only minimally affected. This can be readily seen by the ratio of $CD4^+CD8^{lo}$ to $CD4^-CD8^+$ thymocytes (Figure 6A).

In the analysis of *OT-I* thymocytes, we found that a bivariant plot of TCR (Vα2) versus CD69 did not yield clearly definable subsets, so we instead enumerated the mature thymocytes on the basis of a $V\alpha2^{hi}HSA^{lo}$ phenotype (Figure 6B). Again, we found that the ratio of CD4 to CD8 mature thymocytes decreased with a decreased expression of Erk (Figure 6C, insert box) with one exception. In the Erk1/2-deficient mice, the ratio increased back to wild-type levels. We explain this by noting that DPs in these mice have lower levels of Vα2 and thus probably express endogenous TCR α chains. We conclude that a partial loss of Erk2 in *OT-I* mice had a marked effect on the efficiency of positive selection with less of an effect on the net steady-state number of mature CD8 thymocytes. We have no simple explanation for the reduced-Erk-associated increase in total thymocytes (Figure 6D), although perhaps Erk signaling can play a role in cell death—either negative selection caused by a superoptimal selection signal or death by neglect. Another possibility is that Erk does not mediate cell death, but with decreased Erk, fewer thymocytes have been diverted into a nonproductive CD4 pathway.

Erk1 and Erk2 Both Regulate Egr1 Expression

To investigate the contributions of Erk isoforms to the regulation of downstream target genes, we examined Egr1 induction in DPs with or without the expression of *Erk1/2*. Egr1 is a zinc-finger transcription factor shown to be important to thymocyte selection and is regulated by the MAP kinase pathway (Bain et al., 2001; Bettini et al., 2002). Thymocytes were analyzed for subsets from mice of varying genotypes and the DP thymocytes isolated by cell sorting (Figure 7A). The sorted DP thymocytes were cultured for 45 min in the presence or absence of PMA, and a sample was used to detect the amount of Erk1/2 expressed as a measure of the efficiency of recombination. As shown, the presence or absence of bands corresponding to Erk1 and Erk2 WT matched the genotypes exactly (Figure 7B). As alluded to above, this shows that the cells that complete maturation to the double-positive stage of development are not those that managed to avoid *Erk2* deletion.

Western blotting with anti-Egr-1 revealed a strong induction by PMA after 45 min, and this induction was directly proportional to the amount of expressed Erk (Figure 7B). *Erk1*$^{-/-}$ mice generated approximately the same levels of Egr1 as wild-type, and DP thymocytes from *Erk2*$^{fn/fn}$*;pLck-cre* mice induced substantially less Egr1 protein. Furthermore, Egr1 induction was completely absent in cells from *Erk1*$^{-/-}$*;Erk2*$^{fn/fn}$*;pLck-cre* mice. These results suggest that both Erk isoforms have the ability to regulate Egr1, but under the conditions tested, Erk2 alone provides sufficient signaling to maximally upregulate Egr1.

Discussion

In this report, we describe a newly derived strain of mice in which the *Erk2* gene can be inactivated by the tissue-specific expression of the cre recombinase. In conjunction with a germline mutant allele of *Erk1*, we were able to dissect the role of these two canonical

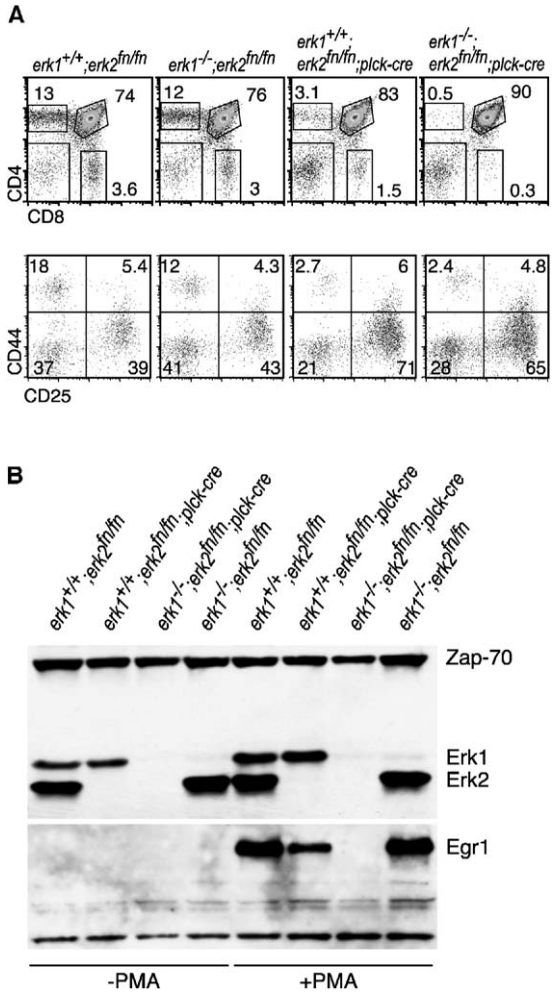

Figure 7. Egr1 Induction in *Erk* Mutant Mice

$CD3^-$ DP thymocytes were cultured for 45 min with or without 10 ng/ml of PMA for 45 min. Lysates of stimulated and unstimulated cells from the indicated mice were analyzed by western blotting for Egr1 expression. ZAP-70 expression is shown as a loading control, and Erk1/2 expression is shown to reveal the levels of Erk protein present in the assayed population. Results are a representation of three independent experiments.

MAP kinases in several aspects of thymic development.

In response to a wide variety of extracellular mediators, Erk is transiently activated, and in many cell types it participates in differentiation, cell-cycle progression, and survival (Pages et al., 1993; Xia et al., 1995; Gardner and Johnson, 1996; Brunet et al., 1999; Walter et al., 2000; Stemmann et al., 2001; Vaudry et al., 2002). In addition, Erk is thought to be essential for the oncogenic activity of Ras and Raf (Wan et al., 2004) and, as such, an important mediator of cell-cycle regulation. Most experiments carried out to characterize the role of Ras-Erk signaling in mammalian cells have relied upon the overexpression of gain-of-function mutants; dominant-interfering forms of Ras, Raf, or Mek; or pharmacological inhibitors of Mek. These experiments have consistently shown that the Ras-Raf-Mek-Erk cascade is important for growth-factor-receptor-mediated mitogenic activity, although individually, each approach has limitations.

In keeping with these results, previous experiments, including our own, showed that proliferation and differentiation associated with the β selection checkpoint are highly dependent on Mek (Crompton et al., 1996; Sharp et al., 1997). To address this issue more conclusively, we analyzed early thymic development in mice deficient for *Erk1/2*; however, because Erk2 was conditionally deleted, there exists the potential for retention of Erk2 protein during differentiation. By comparing the signal in the DN4 subset from *Erk1/2* knockout mice with a titration of cell equivalents from wild-type mice, we found that the decrease in Erk2 is less than 2-fold. Presumably, only those DN4 cells with Erk2 continue to divide, although the same is not true for the DP population. The abundance of DPs allowed us to analyze ten times as many cells, and yet we found no detectable Erk-protein expression (Figure 7). We interpret these data to show that Erk is necessary for proliferation associated with β selection, but not to complete the DN4 to DP differentiation step.

To account for the variability in the expansion of DN3 thymocytes and the retention of Erk2 protein at the DN4 stage, we propose that continued Erk signaling is required through at least the DN4 stage of development. We speculate that the variability reflects the timing of *Erk2* deletion with respect to a productive TCR β chain rearrangement. Those cells that complete a productive rearrangement while retaining a sufficient level of Erk2 are able to sustain proliferation. Such a geometric expansion, sensitive to initial conditions, is likely to exhibit a variable outcome.

The second checkpoint in thymocyte development is positive selection and the associated fate specification resulting in regulated differentiation of CD4 and CD8 T cells. In many unbiased genetic screens for genes involved in fate specification associated with organogenesis, the MAP kinase pathway has been shown to play an essential role. Cells receiving a strong signal originating from a receptor tyrosine kinase adopt a primary cell fate, whereas cells receiving a weaker signal can adopt a secondary fate depending on other signals received (Wassarman et al., 1995; Kayne and Sternberg, 1995).

With these studies as a background, experiments were carried out to characterize the role of Erk signaling in T cell differentiation and lineage commitment. Some studies relied upon the expression of dominant-interfering forms of Ras, Raf and Mek; a hypersensitive Erk mutant; or pharmacological inhibitors of Mek1/2 (Crompton et al., 1996; O'Shea et al., 1996; Swan et al., 1995; Alberola-Ila et al., 1995; Sharp et al., 1997; Bommhardt et al., 1999). Others inhibited signaling with alternations in the TCR (Werlen et al., 2000; Delgado et al., 2000). The results consistently showed a role for the Erk pathway in proliferation and differentiation, yet they were at odds on the issue of lineage commitment. The thymocyte-specific expression of a hypersensitive Erk2 transgene favored CD4 development, and likewise Mek inhibitors favored CD8 development. If CD4 and CD8 expression represent primary and secondary cell fates, respectively, then this would be congruent with the logic of fate specification established for organogene-

sis in simple metazoans. It is also consistent with a model of thymocyte lineage commitment in which the strength of signal through the TCR complex is deterministic (Robey and Fowlkes, 1994; Zamoyska and Lovatt, 2004; Taniuchi et al., 2004; Laky and Fowlkes, 2005). Other results were not consistent with this role for Erk signaling in T cell differentiation. The expression of dominant-interfering forms of Ras and Mek potently inhibited positive selection, and yet there was no apparent imbalance in CD4 and CD8 differentiation.

An analysis of the thymocytes deleted for *Erk1*, *Erk2*, or both further demonstrate that Erk is not merely important, but is essential for positive selection and the differentiation of DP thymocytes to either CD4 or CD8 T cells. Furthermore, none of the more distantly related Erk family members substitute for Erk1 and Erk2. In addition, these results may be revealing with respect to the process of lineage commitment. We observed that the extent of positive selection was proportional to the total amount of Erk present in DPs to the point where there were virtually no CD4 or CD8 SP thymocytes in the absence of both Erk1 and Erk2. We conclude that the level of Erk signaling regulates the number of cells that successfully pass the checkpoint of positive selection. Earlier experiments led to the same conclusion although a similar level of inhibition was achieved by transgenesis, resulting in a 30-fold overexpression of catalytically inactive Mek (Alberola-Ila et al., 1995). Other aspects of these transgenic mice were more difficult to understand because experiments analyzing early thymocyte proliferation or T cell mitogenic responses revealed no effects, whereas preliminary experiments show that genetic ablation of *Erk2* profoundly affects the survival of activated T cells (W. D'Souza, A.M.F., and S.M.H., unpublished observation). We emphasize that all genetic or pharmacological manipulations result in pleiotropic effects, and so understanding comes from multiple independent approaches to a problem.

Consistent with a role for Erk beyond positive selection, we found that CD4 and CD8 thymocyte differentiation exhibited a differential requirement for Erk. The ratio of CD4 to CD8 SP thymocytes decreased with decreasing amounts of Erk whether *Erk2* was deleted early with *Lck-cre* or later with *Cd4-cre*. These results were reinforced by experiments with TCR transgenic mice. Decreasing amounts of Erk had a proportionate effect on the number of CD4 SP thymocytes in AND mice, whereas equivalent losses of Erk had little affect on the number of CD8 SPs in *OT-I* mice. Whether or not the analyses were carried out with TCR transgenic mice, the ratio of CD4 to CD8 SP thymocytes was decreased in proportion to the level of Erk expression.

The major question is whether the level of Erk (and presumably Erk activity) is deterministic for CD4 and CD8 lineage commitment analogous to its role in the models cited above. In the present analysis, we found that a progressive loss of signaling through the MAP kinase pathway was not sufficient, in itself, to cause MHC-class-II-specific thymocytes to assume a CD8 phenotype. This is in contradistinction to studies also using AND TCR transgenic mice in which CD4 was deleted (Matechak et al., 1996), ZAP70 activity was temporally attenuated (Liu and Bosselut, 2004), or Lck activity was diminished with a distal Lck-driven kinase-dead form of Lck (Hernandez-Hoyos et al., 2000). The latter study is most analogous to the work presented here, and yet we note that an earlier analysis with the same dominant-negative *Lck* transgene did not observe a change in lineage commitment (Hashimoto et al., 1996). In addition, diminished TCR signaling achieved via mutations in the TCR zeta chain also did not cause a shift to the CD8 lineage in AND mice (Shores et al., 1997).

One possibility is that the strength of signal affects the probability that DPs will undergo positive selection with a bias, reinforced by selection, toward a given fate (Sharp and Hedrick, 1999; Davis et al., 1993; Leung et al., 2001). Perhaps the present experiments did not detect a lineage reversal because cells that made the decision to become CD8SPs died as a consequence of the associated loss of signaling through CD4. However, this explanation is unsatisfactory because the same caveat should apply to the studies cited above. A second possibility is that the strength of signal *is* deterministic for fate specification, but it is not restricted to Erk activation. It includes other signaling networks such as the release of free calcium and the resulting activation of calcineurin (Neilson et al., 2004). A third possibility is that the strength of signal only determines the probability of positive selection, and lineage commitment is determined by a combination of signal duration (Shao et al., 1999; Wilkinson and Kaye, 2001; Adachi and Iwata, 2002), selection (Leung et al., 2001), or both.

Several lines of experimentation demonstrate the existence of a selection process that could provide a form of proofreading to multiply the accuracy of lineage commitment. This is one interpretation of experiments in which ectopically expressed coreceptors allow for a small population of misdirected T cells (Robey et al., 1994; Davis et al., 1993; Leung et al., 2001). Although "instruction" followed by "selection" would be an attractive model for accurate lineage commitment, Singer and his colleagues explain the available data in the context of a coreceptor reversal model (Singer, 2002). They propose that DP thymocytes receive a positive-selection signal that causes them to exhibit a $CD4^{+}CD8^{lo}$ phenotype, and lineage commitment is secondarily determined by the presence or absence of continued coreceptor signaling. Those cells that receive sustained signals differentiate into CD4s and those for which signaling is attenuated become CD8s. Most convincingly, Sarafova et al. have now shown that the expression of CD4 under control of a CD8 enhancer results in the loss of CD4 expression upon positive selection. In these mice, MHC-class-II-specific thymocytes almost uniformly assume a CD8 cytotoxic phenotype (Sarafova et al., 2005).

We favor a scheme in which the MAP kinase pathway, leading to the expression of Sap-1 (Costello et al., 2004), Egr1 (Bettini et al., 2002) and Id3 (Rivera et al., 2000), promotes positive selection and biases the differentiation toward either CD4 or CD8 depending on the strength of activation. The loss of Erk preferentially diminishes the propensity of progenitors to assume a CD4 phenotype, but this is not sufficient to produce a shift to CD8 T cells. Presently, we cannot say whether the critical step in lineage commitment comes from other signaling pathways or more simply reflects the persistence of signals through the CD4 coreceptor. The

interesting issue will be to determine how signaling and selection combine to affect the expression of transcription factors that appear to program lineage commitment. One such factor is TOX, an HMG-box transcription factor that appears to be conditionally sufficient for CD8 differentiation (Aliahmad et al., 2004). Another is a zinc-finger transcription factor previously known as c-Krox (or Zfp-67), which has been found to determine CD4 versus CD8 differentiation (Keefe et al., 1999; He et al., 2005). It is newly designated as Th-POK, and information pertaining to its induction and the gene expression it elicits downstream may finally provide a molecular explanation for thymocyte lineage commitment.

Experimental Procedures

Mice

Mice harboring the *erk2* exon 3 conditional allele were generated by the UCSD Transgenic Mouse Core Facility as described in Supplemental Experimental Procedures. The *Erk2^{fn}* allele was typed with the primers, 5′-GAACTTACTATGCACATCAGG-3′ (p2 in Figure 1) and 5′-ACACAGTATGAGTCTCATTCC-3′ (p3 in Figure 1). The *Erk2^{f}* allele was typed with the primers, 5′-TAGCAG GTGGATATC TAAGC-3′ (p1 in Figure 1) and 5′-GGTAACAAGAGCAACACGTGC-3′ (p4-Figure 1). *Cd4-cre* and *Lck-cre* mice were obtained from Taconic Labs. *OT-I* mice were a gift from Kristen Hogquist. AND mice were bred in our facility and backcrossed to C57BL/6 for nine generations. All mice were housed at UCSD under institutional guidelines.

FACS Analysis

For total thymocyte analysis, cells were stained with anti-CD69FITC, anti-TCRβPE (H57), anti-CD8PerCP and anti-CD4APC. For analysis of DN-thymocyte populations, cells were stained with a biotin cocktail containing antibodies against CD4, CD8, γδTCR, B220, and MHC class II and then stained with strepavidinPerCP, CD25FITC, and CD44APC. For intracellular β chain staining via H57PE, cells were fixed with Cytofix and permeablized by Cytoperm (BD Pharmingen) as per instructions by the manufacturer. All fluorescent antibodies used in this study were purchased from eBioscience except for PerCP antibodies that were purchased from BD Pharmingen.

BrdU Analysis

Mice were innoculated with 100 μl of a 10 mg/ml BrdU stock intraperitoneally 3 hr before the thymus was harvested. Thymocytes were stained with the same biotin antibody cocktail as stated above and then stained with strepavidin-PerCP, CD25PE, and CD44APC. Cells were placed in BrdU buffer overnight (PBS, 0.01% tween20, 1% paraformaldehyde). One milliliter of fresh 0.1 mg/ml DNase was added and then incubated for 1 hr at 37°C. Cells were stained with 10 μl/10^6 cells of anti-BrdU FITC (BD Pharmingen) for 30 min at room temperature.

Western Analysis

5 × 10^6 thymocytes were lysed with high-salt lysis buffer (20 mM Hepes [pH 7.9], 420 mM NaCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 25% glycerol) with protease and phosphatase inhibitors (Cell Signaling). Supernatants were separated by 10% polyacrylamide SDS gel electrophoresis (BioRad) and transferred to a PVDF membrane (Millipore). Blots were incubated with primary antibody at 4°C overnight and secondary HRP antibody (Vector Labs) for 2 hr. ECL (Amersham) chemistry was used to visualize protein products. Anti-Erk antibodies were from Santa Cruz Biotech (C-14) and Zymed. The anti-Egr1 antibody was obtained from Active Motif, and the ZAP-70 mouse monoclonal antibody was obtained from Transduction Laboratories.

Isolation of Thymocyte Subsets

DN3, DN4, DP, and CD4 thymocytes were sorted by The Scripps Research Institute cell-sorting facility. Western blots were performed with extracts from 10^5 thymocytes, and PCR was performed with 10^4 cells. Deletion of *Erk2* exon 3 was analyzed with p2 and p3 to detect *loxP* sequence and with p1 and p3 to detect to loss of exon 3 (above).

Thymocyte Stimulations

Purification of preselecting DP thymocytes were done by first collecting the $CD3^-$ fraction off of a MACS column (Miltenyi Biotech) and then by collecting the $CD8^+$ fraction. Two milliliters of 2.5 × 10^6 cells/ml of purified preselecting DP thymocytes were plated into a 35 mm dish. Samples were stimulated with 5 μl of a 4 μg/ml PMA stock (CalBiochem) for 45 min. Stimulated and unstimulated samples were washed with 13 ml cold PBS and lysed as described above.

Supplemental Data

Supp data include two Supplemental Figures and Supplemental Experimental Procedures and are available at http://www.immunity.com/cgi/content/full/23/4/431/DC1/.

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