A requirement for sustained ERK signaling during thymocyte positive selection in vivo

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It is unknown how the contrasting events of positive and negative selection can lead to the distinct biological outcomes of life or death. An increasing body of evidence suggests that the duration of extracellular signal-regulated kinase (ERK) signaling plays a role in thymocyte selection. However, it remains unclear what the kinetics of ERK activation are during positive selection in vivo. In this study, we examined the magnitude and duration of ERK signaling in intact murine thymic tissues cultured under conditions of negative or positive selection. We found that negative selection induced a rapid and robust ERK activation that is associated with death, whereas positive selection stimulated a lower intensity and brief ERK activation that quickly declined and then gradually increased and was sustained over several days. The expression pattern of Egr-1 (early growth response-1), a downstream ERK effector, correlates with the biphasic kinetics of ERK during positive selection. Id3 (inhibitor of differentiation/DNA binding 3) also exhibits biphasic kinetics but appeared to be independent of ERK signaling. Furthermore, inhibitors of T cell receptor ligation and ERK activation block maturation of CD8 single-positive thymocytes even when added after 24 h. These results demonstrate that the in vivo duration of ERK signaling must be sustained to support positive selection.

T cell development

The T cell repertoire must be as diverse as possible to recognize a vast array of foreign pathogens yet at the same time remain devoid of self-reactive clones. This apparent dichotomy is established during thymic development through the tightly controlled processes of positive and negative selection. Thymocytes bearing T cell receptors (TCRs) that fail to productively interact with self-peptide–MHC complexes die by neglect. Positive selection ensures self-MHC restriction, whereas negative selection eliminates cells that are specific for self-peptides, ensuring self-tolerance. Thus, ligation of the TCR by self-peptide–MHC complexes results in either death or differentiation of the thymocytes into mature T cells (1). Yet it is unknown how a thymocyte can distinguish between signals that lead to the opposing outcomes of survival and death.

Differential intracellular signals, such as the various mitogen-activated protein kinase (MAPK) cascades, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (Jnk), and p38, could discriminate between positive and negative selection. Studies with knockout mice and specific MAPK inhibitors have demonstrated that Jnk and p38, with the appropriate selection and thymic environment, an intact thymic lobe. We found that ERK activation was biphasic and sustained in intact thymus under positively selecting conditions and robust but transient during negative selection. Furthermore, induction of downstream ERK effectors correlated with the extended duration of ERK induction by positive selection. Prolonged ERK activation and maturation of thymocytes depends on sustained TCR/peptide–MHC interaction and continual ERK phosphorylation.

Materials and Methods

Mice. OT-I is a C57BL/6 transgenic strain expressing a TCR specific for the ovalbumin (OVA) 257–264 peptide in the context of K\(^\alpha\) (20). OT-I TAP (transporter associated with antigen processing) null mice were generated by breeding OT-I transgenic mice to TAP-1\(^{-}\) mice on a C57BL/6 background. Egr-1 (early growth response-1) null mice were generated as described in ref. 21 and were a generous gift from G. Kersh (Emory University, Atlanta). OT-I mice were crossed to Egr-1\(^{-}\) mice to create OT-I-Egr-1\(^{-}\) mice. All mice were treated in accordance with federal guidelines approved by the University of Minnesota Institutional Animal Care and Use Committee.

Peptides and Antibodies. OVAp (SIINFEKL), \(\beta\)-catenin peptide (\(\beta\)CatTp) (RTYTYEKL), and P815p (HYEFPQL) were synthesized by Invitrogen. The MEK inhibitors U0126 (Cell Signaling Technology, Beverly, MA) and PD98059 (Invitrogen) were used to block ERK activation. Purified anti-K\(^\alpha\) (clone Y3) and anti-D\(^b\)© 2005 by The National Academy of Sciences of the USA

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Abbreviations: ERK, extracellular signal-regulated kinase; FTOC, fetal thymic organ culture; DP, CD4\(^{+}\)CD8\(^{+}\); double-positive; SP, single-positive; IEG, immediate early gene; Egr-1, early growth response-1; Id3, inhibitor of differentiation/DNA binding 3; \(\beta\)CatTp, \(\beta\)-catenin peptide; MAPK, mitogen-activated protein kinase; TCR, T cell receptor; MEK, MAPK kinase; OVA, ovalbumin; TAP, transporter associated with antigen processing; APC, antigen-presenting cell; MFI, mean fluorescence intensity.

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(clone 28.14.8) (Maine Biotechnology, Portland, ME) were used to block MHC. For flow cytometry, we used the following antibodies: CD4 (clone RM4-5), CD8α (clone 53-6.7), biotinylated anti-mouse IgG1 (clone A85-1), streptavidin-phycocerythrin, mouse IgG1 isotype, inhibitor of differentiation/DNA binding 3 (Id3) (clone B72-1) (BD Pharmingen), biotinylated anti-rabbit IgG (Vector Laboratories), mouse phospho-p44/42 MAPK (Cell Signaling Technology), normal rabbit IgG, and Egr-1 (clone C-19) (Santa Cruz Biotechnology).

**Results**

**The Kinetics and Duration of ERK Activation During Positive and Negative Selection.** Because the duration of ERK signaling in thymocytes has only been examined in vitro and different studies present conflicting kinetic pictures, we investigated ERK activation in vivo (17, 18). FTOC allows the incubation of intact fetal thymic lobes ex vivo for several weeks (22). The endogenous antigen-presenting cells (APCs), the thymic stroma, and resident bone marrow APCs are all preserved in the intact thymus. We used OT-I TAP³ fetal thymic lobes, because all of the thymocytes in the OT-I TAP³ thymus are preselection CD8⁺CD4⁻ thymocytes due to the inability of TAP³ thymocytes to efficiently present peptides on MHC class I molecules. Thus, we had a homogeneous DP thymocyte population that would respond to exogenous peptide synchronously.

In this system, thymic lobes from gestational day-17 OT-I TAP³ mice were excised and cultured overnight in medium to allow expansion of the DP thymocyte pool. Then, exogenous peptides were added continuously to induce selection. The ligands that induce negative or positive selection have been well defined for OT-I transgenic thymocytes (20, 23). Negatively selecting OVAp induces dulling or down-regulation of the CD4 and CD8 coreceptors, mitochondrial membrane permeability transition, and cell death within 24 h after addition of the peptide (24). In organ cultures, very few DP thymocytes remain by 24 h (Fig. 1A). Addition of a control K⁺-binding peptide, P815p, had no effect on thymocyte maturation in OT-I TAP³ cultures (Fig. 1B). Nonetheless, the TAP³ system is slightly leaky, allowing some selection with endogenous rearrangements as evidenced by the low numbers of CD4 and CD8 single-positive (SP) thymocytes in the no-peptide or P815-stimulated lobes. βCATp is a naturally occurring self-peptide presented by K⁺ that very efficiently mediates positive selection of the OT-I thymocytes (23). Mature CD8 SP thymocytes are apparent 48 h after the addition of βCATp (Fig. 1C) and maximal by 96 h. These data confirm that OVAp induces negative selection in OT-I TAP³ FTOC, whereas βCATp mediates positive selection of CD8 SP thymocytes.

We next measured the kinetics and magnitude of ERK activation induced by these peptides by using flow cytometry with an antibody specific for the phosphorylated forms of ERK-1 and ERK-2 (25). We gated on DP thymocytes so that ERK activation could be solely attributed to the DP population and not APCs or other thymocyte populations. After incubation with the peptides, the thymic lobes were quickly fixed in formaldehyde to preserve the phosphorylation status of ERK. Fig. 2A demonstrates that just a 2-min incubation of intact thymic lobes with OVAp was sufficient to induce a dramatic
increase in phosphorylated ERK compared with the control peptide, P815p. Note that the majority of DP thymocytes displayed increased phosphorylated ERK, suggesting that exogenous peptides rapidly gain full access to APC throughout the lobe. The positively selecting ligand, βCATp, also induced rapid ERK activation, although of a lower magnitude than OVA (Fig. 2A). Hence, the exogenous addition of both positive and negative selection ligands to fetal thymic lobes was able to mediate ERK activation that was recently derived from the double-negative population. Therefore, the duration of ERK activation in FTOC was examined with phorbol 12-myristate 13-acetate (PMA), phosphorylated ERK (Fig. 2B). The control peptide, P815p, did not induce expression of phosphorylated ERK at any time point, nor was pERK observed in DP thymocytes that were recently derived from the double-negative population. However, this scenario appears not to be the case because ERK phosphorylation was similar in both DP and CD8 SP thymocytes (data not shown); therefore, we used a broad gate to include both (Fig. 2A and C). In addition, histogram analysis showed a low level of phosphorylated ERK in all thymocytes and not a bimodal distribution where only a subset expressed phosphorylated ERK, as would be expected if only the new DP cells were phosphorylating ERK (Fig. 2A). Thus, although some of the ERK phosphorylation seen in the second phase could be due to an influx of new DP cells, its presence in both DP and SP populations demonstrates that ERK signaling is sustained. In intact thymic tissue, ERK activation is biphasic and sustained under positively selecting conditions.

The Kinetics of Egr-1 and Id3 Expression Correlate with ERK Activation. Immediate early genes (IEGs) are the direct targets of signaling pathways that accumulate quickly after stimulation. Thus, IEGs might function as cellular sensors for the duration of ERK activation. Indeed, only sustained ERK will stabilize IEGs and lead to their extended expression in some cell types (30). Egr-1 is one such IEG; this transcription factor is induced by phosphorylated ERK (31, 32). In addition, Egr-1 promotes positive selection of both CD4 and CD8 thymocytes (33). Negative selection of the intact OT-I TAP⁺ thymic lobes with OVAp stimulated a very strong yet transient expression of Egr-1 that had completely declined by 24 h (Fig. 3A). Conversely, the positively selecting ligand, βCATp, induced weak initial expression of Egr-1 that peaked between 1 and 2 h after stimulation, but a second phase of Egr-1 expression was observed that was sustained for several days. Thus, this pattern is similar to what was observed with phosphorylated ERK.

Id3, an inhibitor of basic helix-loop–helix proteins, is another IEG and a potential downstream target of ERK (34, 35). Id3−/− mice display a severe defect in the development of CD4 and CD8 thymocytes (36). Both OVA and βCATp cultured thymic lobes induced a brief yet robust expression of Id3 that peaked between 1 and 3 h and then immediately decreased (Fig. 3B). After 24 h, however, the expression of Id3 increased in positively selected lobes and was sustained through 96 h. Thus, the kinetics of Egr-1 and Id3 expression correlated with the biphasic activation of ERK in positively selected thymic lobes.

Id3 Expression Does Not Depend on ERK Activation or Expression of Egr-1. It was previously reported that, in thymocytes stimulated in vitro with phorbol 12-myristate 13-acetate (PMA), phosphorylated ERK activates Egr-1 expression, which in turn activates Id3 expression (33, 37). Interestingly, when U0126, a MEK inhibitor, was added to thymic lobes stimulated with βCATp, Id3 expression was unaffected even though ERK phosphorylation was impaired (Fig. 4). To verify our findings, we examined Id3 protein expression by Western blot. OT-I TAP⁺ thymocytes were stimulated with OVAp in vitro. Fig. 4B demonstrates that the addition of U0126 had no effect on the induction of Id3 protein in thymocytes stimulated with OVAp. To further test whether Id3 depended on ERK activation, we looked at OT-I Egr-1⁺ mice (38). Surprisingly, Id3 up-regulation was unaffected by the lack of Egr-1 or the addition of an ERK inhibitor, implying that Id3 can be regulated independently of either (Fig. 6A, which is published as supporting information on the PNAS web site). Finally, the βCATp induction of Id3 was monitored in FTOC with the MEK inhibitor U0126. The inhibitor had no effect.
Expression of Egr-1 and Id3 is biphasic in positively selected thymocytes and transient in negatively selected thymocytes. Egr-1 (A) or id3 (B) expression in OT-I TAP° fetal thymic lobes incubated with OVAp, βCATp, or P815p for the indicated times. DP thymocytes were gated and analyzed for the expression of Egr-1 or Id3 by intracellular staining. The data are expressed as the MFI. Data are representative of at least three independent experiments.

**Fig. 3.** Expression of Egr-1 and Id3 is biphasic in positively selected thymocytes and transient in negatively selected thymocytes. Egr-1 (A) or id3 (B) expression in OT-I TAP° fetal thymic lobes incubated with OVAp, βCATp, or P815p for the indicated times. DP thymocytes were gated and analyzed for the expression of Egr-1 or Id3 by intracellular staining. The data are expressed as the MFI. Data are representative of at least three independent experiments.

ERK Activation Is Required for Positive Selection. To examine whether positive selection of OT-I TAP° CD8 SP thymocytes requires sustained ERK activation, fetal thymic lobes were incubated with U0126 at the beginning of peptide addition or after 24 h. At 96 h, the efficiency of positive selection was determined. βCATp induces efficient positive selection of OT-I thymocytes (Fig. 5A). The control peptide, P815p, was unable to mediate selection of SP thymocytes. When the inhibitor was present from the beginning of the culture, maturation of CD8 SP thymocytes was completely blocked, similar to lobes cultured with P815p. However, even when U0126 was added 24 h after βCATp, positive selection was impaired, implying that the initial burst of phosphorylated ERK is not sufficient for selection and that the second sustained phase of ERK activation is required for maturation of CD8 SP thymocytes (Fig. 5A).

It is possible that the transient nature of the initial ERK activation might be due to down-regulation of the TCR in response to the selecting stimulus. A decrease in the amount of TCR on the DP thymocytes could terminate signaling, preventing the phosphorylation of ERK. Although stimulation with βCATp did induce a modest (50%) decrease in TCR expression, the levels quickly returned (by 6 h), after which the amount of TCR on the surface of the βCATp-stimulated thymocytes continued to increase through 96 h (Fig. 7, which is published as supporting information on the PNAS web site). Although these experiments do not strictly rule out the possibility, it seems unlikely that TCR down-regulation accounts for the rapid loss of ERK activation within the first 24 h.

**Fig. 4.** Id3 up-regulation does not depend on ERK activation or expression of Egr-1. (A) Expression of Id3 and phosphorylated ERK in OT-I TAP° fetal thymic lobes. The lobes were incubated with βCATp with or without the addition of 20 μM U0126 (MEK inhibitor), gated on DP thymocytes, and analyzed for induction of phosphorylated ERK at 2 min and expression of Id3 at 1 h. The MFI s of three lobes per time point were averaged, and the error bars represent the standard deviation. (B) Western blot of Id3 protein expression in OT-I TAP° thymocytes. Thymocytes were stimulated with OVAp-pulsed B6.5J splenocytes with or without the addition of 20 μM U0126 for 3 h. DP thymocytes were separated by magnetic-activated cell sorting bead separation, and whole-cell lysates were prepared. Expression of Id3 was analyzed in Western blots by using an id3 antibody. Blots were probed with anti-ERK2 antibodies to control equal loading of protein. The numbers below the blot indicate the fold induction of Id3 expression.

ERK Activation Depends on TCR/Peptide–MHC Interaction. To determine whether continued TCR ligation was required for the sustained ERK activation and for positive selection, we blocked the TCR/peptide–MHC interaction with an antibody against the selecting MHC, Kb. This antibody effectively blocks the productive interaction between the Kb molecule and the OT-I TCR without activating the T cell or transducing any signals. Thymic lobes were incubated with βCATp or P815p in the presence or absence of anti-Kb or a control MHC antibody, anti-Dp. Two minutes after stimulation, the lobes were analyzed for ERK signaling. Lobes incubated with βCATp alone or βCATp with anti-Dp induced a vigorous activation of ERK at 2 min (Fig. 5B). The addition of anti-Kb to the lobes prevented ERK phosphorylation, as expected, indicating that ERK activation depends on TCR signaling at the early phase.

To determine whether TCR interaction is required for positive selection, we measured SP development in the presence of anti-MHC antibodies after 96 h. Thymocytes that were cultured in the presence of the Kb MHC blockade for the full 96 h failed to mature into CD8 SP thymocytes (Fig. 5C), whereas those thymocytes incubated with the control MHC antibody underwent positive selection similar to lobes stimulated with βCATp alone. Interestingly, the initiation of MHC blockade 24 h after peptide stimulation...
tation, we measured ERK phosphorylation at 72 h. ERK phosphorylation was decreased in thymic lobes given anti-K\(\text{d}\) at 24 h compared with those that were \(\beta\)CATp-stimulated alone at 72 h (Fig. 5D). Consequently, DP thymocytes depend on continued TCR/peptide–MHC interaction for sustained ERK activation and positive selection.

Discussion

In this study, we used an organ culture system coupled with intracellular staining to evaluate the duration of ERK signaling during thymocyte development in the intact microenvironment. Use of the OT-I system, with defined selecting ligands, allowed us to study a synchronous DP thymocyte population under positive or negative selection conditions. Negative selection induced a strong yet very brief activation of ERK, which peaked at 2 min. Positive selection induced an initial transient ERK activation, which was followed by a low level of ERK phosphorylation that was sustained for 96 h. The kinetics of expression of two downstream transcription factors, Egr-1 and Id3, correlated with the biphasic sustained ERK activation. Inhibition of the second phase of ERK signaling either by blocking ERK activation or inhibiting TCR interaction with peptide–MHC prevented maturation of CD8 SP thymocytes, indicating that the extended duration of ERK was necessary for positive selection.

Our in vivo results are different from those observed in vitro, which portrayed ERK activation lasting only a few hours (17, 18). In both cases, positive selection stimulated a gradual increase in phosphorylated ERK. Our data illustrate that positive selection triggers robust ERK activation within 2 min, which then quickly decreases (Fig. 2B). Only after 24 h of continual stimulation did ERK activation begin to increase again and remain at a steady, persistent level for several days (Fig. 2C). The different kinetic picture we observed might be attributed to the fact that the selecting ligands were presented by endogenous APCs within the context of an intact thymic stroma that was not reaggregated or otherwise disrupted. In addition, we used intracellular staining for ERK activation; others looked at protein levels by Western blotting. Use of flow cytometry allowed us to examine cells on an individual level and to exclude double-negative thymocytes and APCs.

What is the role of sustained ERK activation during positive selection? Recent data demonstrated that ERK can mediate the phosphorylation of Bim (39). Bim is a member of the BH3-only Bcl-2 family that functions to promote cell death by binding to and antagonizing the antiapoptotic proteins Bcl-2 and Bcl-XL and by activating downstream proapoptotic factors Bax and Bak (40). Thymocytes from Bim-deficient mice are less sensitive to apoptosis, indicating a role for Bim in negative selection (41). ERK mediates phosphorylation of Bim on three serine sites that prevent its interaction with Bax and thus its ability to promote cell death (39). Other studies have shown that Bim phosphorylation by ERK leads to its ubiquitination and proteasomal degradation (42, 43). Therefore, one can imagine that the sustained ERK activation that occurs during positive selection could phosphorylate Bim and prevent its proapoptotic activities, allowing maturation of the thymocytes.

It takes DP thymocytes ~3–4 days to complete positive selection. Although other accessory signals from thymic stroma are also required, sustained TCR interaction with peptide–MHC is required for efficient positive selection (44). We have demonstrated that interruption of TCR signaling after the first 24 h can greatly impair positive selection (Fig. 5C). Even if signaling is blocked 72 h after the initial stimulation, maturation of SP thymocytes is partially obstructed (data not shown). Our data clearly show that a thymocyte must be able to interact with its self-peptide–MHC molecule for several days, but we do not know whether this interaction must be continual with a single APC or whether a series of interactions with the appropriate selecting peptide on different APCs is sufficient. A recent study estimated that the mean interaction time between a thymocyte and a stromal cell was at least 30 min during positive selection (45). Because DP thymocytes must migrate from the cortex to the medulla, it is likely that a thymocyte would eventually disrupt this interaction, but when this migration occurs after the initiation of positive selection is not known.

How does positive selection induce two phases of ERK activation? It could be an indirect consequence of TCR down-regulation. The transient nature of the initial ERK activation might be due to down-regulation of the TCR in response to the selecting stimulus. However, \(\beta\)CATp does not induce a very substantial down-regulation of TCR (Fig. 7). It is more likely that the biphasic nature
ERK activation could be an effect of the phosphatases that regulate ERK activity. MAPK phosphatases specifically inactivate MAPKs by removing phosphates from the threonine and/or tyrosine residues in their activation loops (46, 47). The majority of MAPK phosphatases are either constitutive cytosolic phosphatases or inducible nuclear phosphatases. According to the sequential duration, and location of MAPKs (48). Several phosphatases have been identified that have specificity for ERK, including the inducible nuclear phosphatases, and activates many transcription factors. After stimulation by either OVAp or βCATp, ERK is rapidly phosphorylated and peaks at 2 min, then quickly declines (Fig. 2). This rapid activation might be too fast to allow complete translocation to the nucleus, and the constitutive cytosolic phosphatases could dephosphorylate ERK.

Unlike ERK and Egr expression, the initial peak of Id3 expression was identical between OVAp and βCATp, suggesting that a MAPK-independent pathway might influence Id3 expression in vivo. Bone morphogenetic protein (BMP) and Notch have both been implicated in the regulation of the Id family of proteins (50, 51). In fact, the MAPK pathway was not found to regulate Id proteins in other diverse cell types examined, such as B cells (TGF-β), embryonic stem cells (BMP), chondrocytes (BMP), or Xenopus embryos (Notch and BMP) (51–56). Our data recommend further study of the regulation of Id3 during the process of positive selection.

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Previous studies examining the role of Id3 in thymocyte selection concluded that Id3 was downstream of ERK signaling and was affected by the pharmacological inhibition of ERK (33, 37). In contrast, we have found that up-regulation could occur independently of either Egr-1 or ERK activation (Fig. 5). The previous reports examined transcription of Id3 mRNA by Northernblots using artificial stimulation; we looked at Id3 protein levels and stimulation by endogenous self-peptide–MHC. It is possible that posttranscriptional mechanisms lead to increased Id3 protein (49).

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