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Non-redundant function of the MEK5–ERK5 pathway in thymocyte apoptosis

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The mitogen-activated protein kinases (MAPKs) ERK1/2, p38, and JNK are thought to determine survival-versus-death fate in developing thymocytes. However, this view was challenged by studies using 'MEK1-ERK1/2-specific' pharmacological inhibitors, which block both positive and negative selection. Recently, these inhibitors were also shown to affect MEK5, an upstream activator of ERK5, another class of MAPK with homology to ERK1/2. To define the contribution of the MEK5–ERK5 pathway in T-cell development, we retrovirally expressed dominant-negative or constitutively activated form of MEK5 to inhibit or activate the MEK5–ERK5 pathway. We demonstrate that MEK5 regulates apoptosis of developing thymocytes but has no function in positive selection. ERK5 activity correlates with the levels of Nur77 family members but not that of Bim, two effector pathways of thymocyte apoptosis. These results illustrate the critical involvement of the MEK5–ERK5 pathway in thymocyte development distinct from that of ERK1/2 and highlight the importance of the MAPK network in mediating differential effects pertaining to T-cell differentiation and apoptosis.

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Introduction

During development, precursor thymocytes are guided through a number of checkpoints to ensure generation of sufficient numbers of T cells bearing functional antigen receptors that do not cross-react with self-antigens (review by Starr *et al*, 2003). Establishing a proper T-cell compartment requires testing individual T-cell receptors (TCRs) based on their specificity and avidity of interaction with the ligand. At the immature $CD4^+CD8^+$ double-positive (DP) stage, T cells are intrinsically sensitive to apoptosis and they are destined to 'death-by-neglect'. However, low-avidity interactions with self-major histocompatibility complex (MHC) proteins reverse this fate, signalling the T cell to survive and differentiate further (positive selection). High-avidity interactions with peptides derived from self-antigen presented in the context of self-MHC induce apoptosis (negative selection). These developmental checkpoints are critical for the generation of T cells that can later respond to peptides derived from foreign antigens without mounting an autoimmune response to self-antigens. Positive and negative selections also set activation thresholds for a given T-cell repertoire by selecting for cells that bear receptors whose avidity of interaction falls within a narrow permissive range. The result is that as a population, reactivity of mature T cells is controlled, and precious niches are not overtaken by a small number of hyper-reactive clones.

Consequently, it is natural that TCRs have a crucial function in cell-fate decisions during T-cell development. Engagements of TCRs at the cell surface regulate cell-fate determination through stimulation of intracellular molecular pathways. Both positively and negatively selecting ligands activate membrane-proximal events similarly, requiring activation of protein tyrosine kinases $p56^{lck}$ (Lck) and ZAP70. However, the differences in the nature of the interactions between the TCR and the ligand are propagated through downstream events, at least in part through Grb2 and mitogen-activated protein kinase (MAPK) pathways (for review, see Sohn *et al*, 2003). Evidence suggests that negatively selecting ligands induce 'strong' signals that recruit numerous Grb2 adaptor proteins to the plasma membrane and strongly activate the ERK1/2, p38, and JNK MAPKs and apoptosis. In contrast, positively selecting ligands induce 'weak' signals and recruit fewer molecules of Grb2, thus activating only ERK1/2 and leading to T-cell survival (Daniels *et al*, 2006).

This model was initially posited to explain the phenotypes of mouse models in which selective MAPK pathways were inhibited by overexpression of dominant-negative MAPK activators or by gene disruption. For example, expression of dominant-negative forms of the components of the ERK1/2 pathway, or targeted disruption of the genes encoding ERK1 and ERK2 (Fischer *et al*, 2005), leads to a specific block in positive selection. In contrast, overexpression of a dominant-negative form of JNK, or deletion of the *jnk1* or *jnk2* gene, inhibits negative selection but not positive selection (for review, see Sohn *et al*, 2003). In other studies, the requirement for individual MAPK pathways in thymocyte selection was evaluated *in vitro* using chemical inhibitors that block selective MAPK pathways. Use of these inhibitors was believed to circumvent the main caveat of the mouse models; that the dominant-negative effect in transgenic models may have been incomplete. These studies showed that the inhibitors of MEK1/2 (blocking the ERK1/2 pathway) impaired both positive and negative selection (Mariathasan *et al*, 2000, 2001), suggesting that ERK1/2 contribute to the total MAPK signal output in determining the survival-versus-death outcome.

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It was recently shown that at higher concentrations, most pharmacological inhibitors of MEK1/2 also blocked ERK5 activation, presumably through its upstream activator MEK5 (Mody *et al*, 2001). ERK5 represents a novel class of MAPK possessing a unique C-terminal transcriptional co-activator domain in addition to a consensus MAPK catalytic domain in its N terminus (Kasler *et al*, 2000). The catalytic domain of ERK5 most closely resembles ERK1/2 but is activated through phosphorylation exclusively by MEK5 at the TEY dual phosphorylation motif (Kato *et al*, 1997). The C-terminal domain contains a region that mediates protein–protein interactions with the MEF2 transcription factor, as well as a distinct region that functions as a transcriptional co-activator (for review, see Sohn *et al*, 2003). Coordinated regulation of the N- and C-terminal domains permits ERK5 to tether itself as a potent regulator of transcription. This function of ERK5 through MEF2 has been characterized in detail in the context of the *nur77* and *lklf* genes (Kasler *et al*, 2000; Sohn *et al*, 2005). In addition, ERK5 has been shown to activate MEF2 proteins by phosphorylation at a serine residue found in the MEF2 transactivation domain (Kato *et al*, 1997). Interestingly, the ability of ERK5 to phosphorylate MEF2 is shared by the p38 MAPK, and as a result, ERK5 and p38 are likely to regulate an overlapping set of target genes. Given these structural and functional characteristics, ERK5 may perform a unique spectrum of biological functions by affecting pathways related to both ERK1/2 and p38.

Previously, we and others showed that disruption of the *erk5* gene in mice resulted in severe angiogenic defects and embryonic lethality at mid-gestation (Regan *et al*, 2002; Sohn *et al*, 2002; Yan *et al*, 2003; Hayashi *et al*, 2004). Comparative gene profile analyses further pointed to a role of ERK5 in multiple gene response pathways pertinent to both vascular and haematopoietic differentiation and function (Sohn *et al*, 2005). In this study, we present data demonstrating the requirement for the MEK5–ERK5 pathway in T-cell development. We show that ERK5 is activated rapidly and transiently in thymocytes upon TCR engagement. Using retroviral transduction to overexpress dominant-negative or constitutively active MEK5, which exerts restricted specificity towards ERK5, in re-aggregated fetal thymic organ culture (RTOC) and haematopoietic stem cell (HSC) reconstitution systems, we show that MEK5 has a critical function in TCR-mediated apoptosis, although having a minimal function in positive selection. Furthermore, we demonstrate that ERK5 regulates Nur77 and its related family member Nor-1 but not Bim expression in thymocytes, two of the main effector pathways of apoptosis in cells undergoing negative selection (Bouillet *et al*, 2002; Winoto and Littman, 2002). Thus, MEK5→ERK5→MEF2→Nur77 family represents an important signal-transduction pathway of T-cell development.

Results

TCR engagement activates ERK5

ERK5 is exclusively activated by MEK5, which phosphorylates the threonine and tyrosine residues of the TEY dual phosphorylation motif (Kato *et al*, 1997). ERK5 activation occurs in response to a variety of stimuli, including EGF (Kato *et al*, 1998), serum (Kato *et al*, 1997), and hypoxia (Sohn *et al*, 2002). To determine whether TCR stimulation also activates ERK5 in immature T cells, we stimulated pre-selection thymocytes from MHC-deficient (lacking both classes I and II) mice with anti-CD3 and anti-CD28 antibodies and monitored the kinetics of the response. As shown in Figure 1A, ERK5 is phosphorylated within minutes following stimulation, the response peaking at 10 min and declining through the 2-h time point. This response was dose dependent because stimulation with a lower concentration of anti-CD3 antibody shortened the duration as well as a delay in the peak response (Figure 1A). The total abundance of ERK5 did not change over the time course we examined. The rapid response of ERK5 phosphorylation also mirrored the known pattern of phospho-ERK1/2 response, although duration of the ERK5 response was much more transient than ERK1/2 response at the lower anti-CD3 concentration (Figure 1A, top and bottom panels).

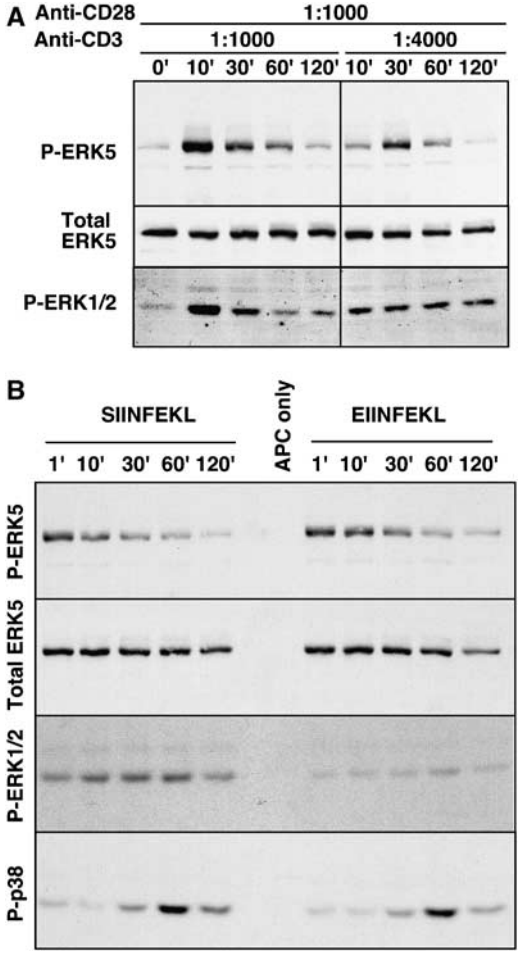

Figure 1 ERK5 activation in thymocytes. (**A**) Activation of ERK5 was assessed in OT-1 MHC-null thymocytes stimulated with anti-CD28 antibodies (at 1:1000) and two different concentrations (at 1:1000 and 1:4000) of anti-CD3 antibodies. Activated ERK5 or ERK1/2 was detected by immunoblotting with anti-phospho-ERK5 antibodies (top panels) or anti-phospho-ERK1/2 (lower panels). Total amounts of ERK5 proteins remain unchanged over the time course (middle panels). (**B**) ERK5 is activated rapidly in response to strong agonist (SIINFEKL) and weak agonist/antagonist (EIINFEKL) peptides. OT-1 MHC-null thymocytes stimulated with glutaraldehyde-fixed EL4 cells pre-coated with SIINFEKL at 10^{-12} M or with EIINFEKL at 10^{-8} M were analysed for activation of ERK5, ERK1/2, and p38. 'APC only' lanes represent peptide-coated, fixed EL4 cells. Total ERK5 levels did not change over the time course.

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We further tested whether peptide antigens stimulated ERK5. In particular, we were interested to see whether the ERK5 response more closely resembled the responses of MAPKs that mediated positive selection (ERK1/2) or those that mediated negative selection (p38 and JNK), using peptides that induce negative (strong agonist) versus those that induce positive (weak agonist/antagonist) selection (Hogquist *et al*, 1994). To address this, we stimulated thymocytes from MHC-deficient OT-1 Rag+ TCR transgenic mice (consisting mostly of pre-selection DP cells) with glutaraldehyde-fixed antigen-presenting cells pre-coated with SIINFEKL (strong agonist) or EIINFEKL (weak agonist) peptide. We chose peptide concentrations that were shown previously (Alberola-Ila *et al*, 1996; Daniels *et al*, 2006) and confirmed by us (data not shown) to induce comparable levels of CD69 surface expression. Thus, SIINFEKL at 10^{-12} M and EIINFEKL at 10^{-8} M, respectively, induce approximately equivalent total levels of TCR signal. Under these 'quantitatively equivalent' conditions, we compared MAPK responses to define any qualitative differences. Figure 1B shows that ERK5 is activated rapidly, with phosphorylation occurring within 1 min (the signal for unstimulated cells is similar to time point 0 in Figure 1A) and declining over the course of 2 h. This rapid and transient activation kinetics is consistent with the known pattern of MAPK response in stimulated T cells (Delgado *et al*, 2000; Werlen *et al*, 2000); however, the peak response of ERK5 activation appeared to temporally precede those of ERK1/2 and p38 (Figure 1B). This suggests that the activation threshold for ERK5 may be lower compared to other MAPKs and that ERK5 activation is extremely sensitive to signals generated by TCR engagement. This is further supported by our observation that phospho-ERK5 response to TCR crosslinking becomes saturated at lower concentrations of anti-CD3 than phospho-ERK1/2 response (Supplementary Figure 1). We also noticed that the activation profiles of ERK5 and p38 were distinct from that of ERK1/2 in that ERK1/2 response, as reported in the literature (Werlen *et al*, 2000; McNeil *et al*, 2005), was overall stronger to SIINFEKL (stronger intensity of the signal) than to EIINFEKL, whereas p38 and ERK5 responded similarly to both the strong and weak agonists. These results allowed us to categorize ERK5 and p38 together, despite differences in their activation kinetics, because their responses were driven by the quantitative component of the TCR signal. This is in contrast to ERK1/2, which has been shown to respond differently to positively versus negatively selecting peptides (Werlen *et al*, 2000; Mariathasan *et al*, 2001; McNeil *et al*, 2005).

MEK5 activity levels influence survival of the DP thymocytes

MEK5 is an exclusive upstream kinase for ERK5, and MEK5 mutant embryos exhibit identical defects as do ERK5 mutant embryos (Wang *et al*, 2005). Overexpression of MEK5(A) or MEK5(D) has been shown previously to inhibit or activate the catalytic activity of ERK5, respectively (Kato *et al*, 1997, 1998). Thus, we generated retroviral constructs that encode a dominant-negative form of MEK5, MEK5(A), or an activated form of MEK5, MEK5(D), and used them to assess the function of ERK5 in T cells (Figure 2A). These constructs also encode GFP under the control of the internal ribosomal entry site (IRES), allowing us to correlate the effects of ERK5 inhibition or constitutive activation on T cells with GFP expression.

To confirm the effects of MEK5(A) or MEK5(D) expression on ERK5 signalling, we first evaluated the ERK5 response following T-cell stimulation. T-cell hybridomas were used because adult thymocytes were difficult to infect, and their low viability and cell number limited the feasibility of further analysis by western blotting. ERK5 phosphorylation at threonine 218 and tyrosine 220 was assessed using phospho-specific antibody (Figure 2B, upper panel) and by comparing the relative signal intensities of the slower migrating band (activated) to the faster migrating band (unactivated) in total ERK5 western blot (lower panel). ERK5 activation is suppressed by MEK5(A) as evidenced by the lack of conversion from the faster to slower migrating band in the total ERK5 blot (Figure 2B, lower panel) and the decreased signal intensity of the phospho-ERK5 bands in activated T cells (Figure 2B, upper panel). Conversely, MEK5(D) prolonged the duration of the phospho-ERK5 signals and correspondingly increased the signal intensity of the upper band compared to the lower band (Figure 2B). Although augmentation of ERK5 phosphorylation at Thr218/Tyr220 in MEK5(D)-expressing cells appeared to be activation dependent (Figure 2B, lower panel), this could be due to the lower sensitivity of this antibody because ERK5 was phosphorylated in MEK5(D)-transfected cells prior to stimulation (at $t=0$), as indicated by the constitutive presence of the slower migrating band in the total ERK5 immunoblot (lower panel). Alternatively, activation-inducible factors, in addition to MEK5(D), might further increase ERK5 phosphorylation and its full activation. We also evaluated the expression levels of known ERK5 target genes in activated DO11.10 hybridoma cells expressing MEK5(A) or MEK5(D). As shown in Figure 2C, two of the known ERK5 target genes, *nur77* (Kasler *et al*, 2000) and *lklf* (Sohn *et al*, 2005), responded to changes in MEK5–ERK5 activity. MEK5(A) decreased, and MEK5(D) increased, the expression levels of *nur77* and *lklf*. MEK5(A) also inhibited induction of another Nur77 family member, *nor-1*. In contrast, the levels of *bim*, or an ERK1/2 target gene, *egr-1*, did not correlate with MEK5–ERK5 activity consistently (Figure 2C). These results were also confirmed at the protein level for Nur77, Nor-1, and Bim (data not shown). Thus, MEK5(A) and MEK5(D) specifically inhibit and activate the ERK5 pathway, respectively.

We next evaluated the effect of MEK5(D) in adult mouse thymocytes by transient transfection using the Amaxa technology (www.amaxa.com). Because of the low transfection efficiency and low thymocyte viability post-transfection, it was not possible to assess the effect of MEK5(A) nor was it possible to perform western blot analysis. Transfected thymocytes were stimulated with anti-CD3 and anti-CD28 antibodies *in vitro* because full activation of ERK5 by MEK5(D) still required TCR stimulation (as shown in Figure 2B), before analyses by quantitative RT–PCR. Consistent with the results in DO11.10 cells, the levels of both *nur77* and *nor-1*, and that of *lklf*, increased with MEK5(D), showing a strong correlation between expression of the Nur77 family and total MEK5 and ERK5 activities (Figure 2D). In contrast, the level of *bim*, which also has been shown to be inducible following TCR stimulation (Bouillet *et al*, 2002) and has an important function in negative selection through Bak and Bax, was not induced by MEK5(D) (Figure 2D). The level of *egr-1* also remained unaffected.

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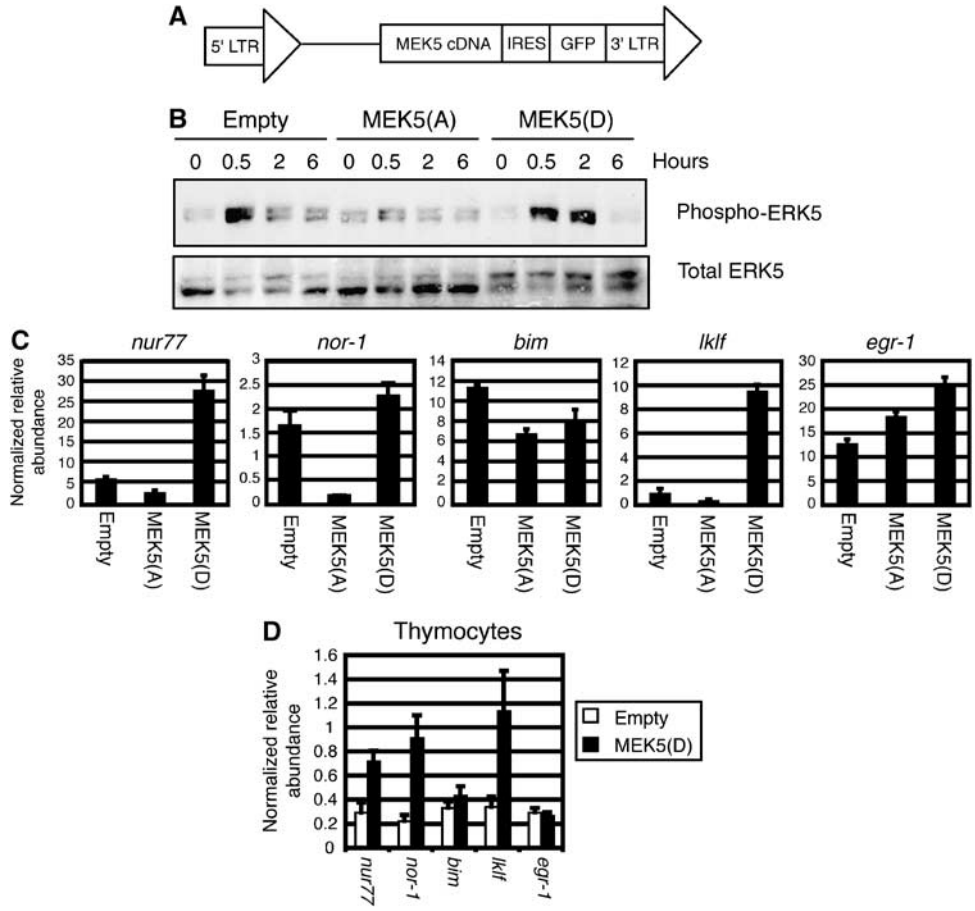

Figure 2 Expression of dominant-negative or activated MEK5 in T cells. (**A**) Representation of the retrovirus construct for expression of MEK5(A) or MEK5(D). This construct drives co-expression of GFP under IRES, allowing identification of cells that are retrovirally transduced. (**B**) The effects of MEK5(A) and MEK5(D) overexpression on ERK5 activation responses. Total lysates from empty, MEK5(A), or MEK5(D) retrovirus-transduced DO11.10 hybridoma cells were analysed by immunoblotting for phospho-ERK5 (upper) or total ERK5 (lower) at indicated time points following stimulation with ionomycin at 0.5 μM and PMA at 2.5 ng/ml. MEK5(A) inhibits, whereas MEK5(D) enhances, ERK5 activation. (**C**) The effects of MEK5(A) and MEK5(D) on ERK5 target gene expression in T cells. Retrovirally transduced DO11.10 hybridoma cells were stimulated for 6 h with ionomycin and PMA and were analysed by quantitative RT–PCR for expression of known ERK5 target genes (*nur77* and *lklf*), apoptotic gene (*bim*), a Nur77-related family member (*nor-1*) or ERK1/2 target gene (*egr-1*). Relative values were calculated by normalization against corresponding HPRT values and expressed as the mean. Error bars indicate standard deviation of triplicate samples. Data from a representative experimental set are shown. (**D**) Responses of *nur77*, *nor-1*, *bim*, *lklf*, and *egr-1* genes to activated ERK5 in primary thymocytes. Thymocytes were transiently transfected with pCI (empty) or pCI-MEK5(D), stimulated *in vitro* with anti-CD3 and anti-CD28 antibodies for 4 h and analysed by quantitative RT–PCR. These values were normalized against HPRT. The average values of triplicate samples are shown, with the error bars indicating standard deviation. Results representative of three independent experiments are shown.

To assess the contribution of the MEK5–ERK5 pathway in thymocyte differentiation, we employed a RTOC system using retrovirally transduced fetal thymocytes (Hernandez-Hoyos *et al*, 2003). Correlating with their effects on expression levels of apoptotic genes *nur77* and *nor-1*, MEK5(A) and MEK5(D) exerted opposite effects on the relative accumulation of GFP^+ thymocytes in the RTOC system. Starting with the same number of cells at the time of infection, expression of MEK5(A) consistently increased ($P<0.05$) the representation of GFP^+ cells, compared to empty virus control (Figure 3A, left panel). In contrast, expression of MEK5(D) dramatically decreased the number of GFP^+ cells. This result suggested that MEK5(A) enhanced, whereas MEK5(D) inhibited, cell survival and/or expansion in developing thymocytes. The effect was predominantly exerted at the DP and not DN stage (Supplementary Figure 2A). Consistent with this idea, the percentage of apoptotic cells among DP thymocytes increased with MEK5(D) and slightly decreased with MEK5(A) expression in these RTOC lobes (Figure 3A, right panel).

Furthermore, MEK5(A) expression consistently increased the percentage of GFP^+ DP cells in the re-aggregated cultures compared with empty virus control (Figure 3B, average of empty virus control $= 28.40 \pm 3.45\%$ ($n=8$); average of MEK5(A) $= 43.72 \pm 6.47\%$ ($n=6$); $P<0.05$). Because the absolute cell number of the MEK5(A)-infected population was also higher, this translated to an increased number of DP thymocytes that accumulated with inhibition of ERK5 (Figure 3E, lower panels). In contrast, MEK5(D) dramatically reduced the total GFP^+ cell number and also decreased the proportion of DP cells (Figure 3B; note: most of the cells die with MEK5(D) retrovirus transduction and thus hardly any events were registered in the flow profile). These differences reflected gene-specific effects because the GFP^- counterparts displayed comparable CD4 and CD8 expression profiles (Supplementary Figure 3A). We also observed a small reduction in the percentage of $CD4^+$ single-positive (SP) T cells among the MEK5(A)-expressing population (average of empty virus control $= 15.91 \pm 2.64\%$ ($n=7$); average of

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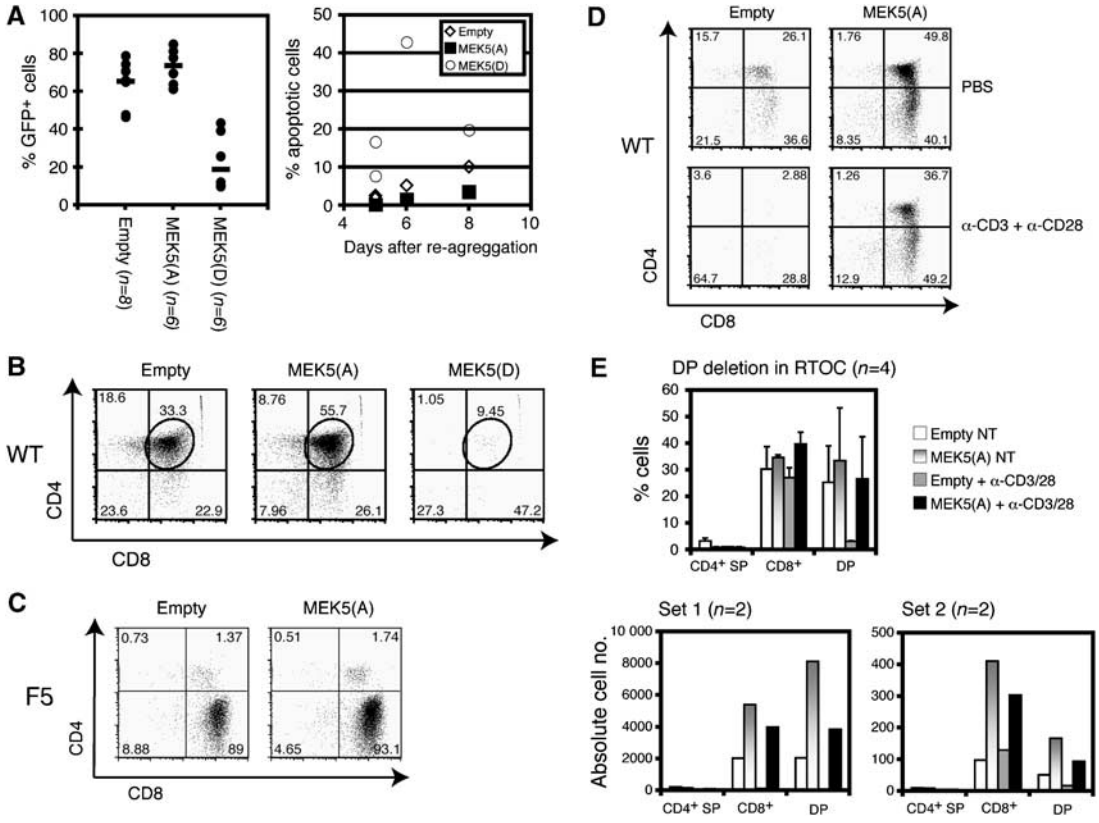

Figure 3 Differentiation of thymocytes in RTOCs. (**A**) Relative ERK5 activity correlates with apoptosis of DP thymocytes in RTOCs. Viability of RTOC thymocytes expressing empty virus, MEK5(A), or MEK5(D) was evaluated by relative representation of GFP$^+$ cells (left panel) and annexin V$^+$ (apoptotic) cells (right panel) in the CD4$^+$CD8$^+$ DP compartment. ERK5 inhibition correlates with enhanced accumulation of thymocytes (percent GFP$^+$, the bar indicating the mean value) and decreased cell death (percent annexin V$^+$, each symbol representing individual RTOC lobes at various days after re-aggregation), whereas ERK5 activation correlates with reduced representation of GFP$^+$ cells and increased apoptosis. (**B**) Differentiation of RTOC thymocytes transduced by empty virus, MEK5(A), or MEK5(D) retrovirus was analysed by CD4 and CD8 expression of the GFP$^+$ population. The numbers correspond to percentages of cells in individual gates. MEK5(A) increased, whereas MEK5(D) decreased, the representation of DP thymocytes in these RTOCs. The percentages of cells in the MEK5(D) profile include events that have accumulated against the *x* axis and are therefore difficult to see. (**C**) Positive selection is unimpeded in thymocytes expressing MEK5(A). The effect of ERK5 inhibition on positive selection was evaluated by monitoring maturation of F5 Rag+ transgenic thymocytes transduced with empty virus or MEK5(A) virus in RTOCs. At 9 days after re-aggregation, RTOC lobes were disrupted and analysed by CD4 and CD8 staining, gated on live GFP$^+$ population. (**D**) MEK5(A) inhibits TCR-mediated deletion of thymocytes. RTOCs re-aggregated with empty virus- or MEK5(A) virus-infected thymocytes were treated with PBS (upper panels) or anti-CD3 and anti-CD28 antibodies (each at 1:1000 ascites dilution, lower panels) overnight and analysed by CD4 and CD8 staining. Representative CD4/8 profiles of GFP$^+$ population from two independent experiments are shown. (**E**) The protective effect of MEK5(A) in TCR-mediated deletion. The response of empty virus- or MEK5(A) virus-transduced thymocytes in the CD4$^+$ SP, CD8$^+$, and DP compartments are shown as percentages (upper) or cell numbers (lower) following treatment with media alone (NT) or with anti-CD3 and anti-CD28 antibodies. For the cell number data, results from two independent experiments are shown separately. The numbers differ widely between experiments due to the differing efficiency of RTOC in culture.

MEK5(A) = 6.88 ± 2.16% ($n = 7$); $P < 0.05$). However, experiments utilizing TCR transgenic mice (see below) showed no appreciable effect of MEK5(A) in positive selection. Taken together with the results correlating increased apoptosis with MEK5(D) expression and decreased apoptosis with MEK5(A) expression (Figure 3A), these data suggest that activation of the MEK5–ERK5 pathway induces apoptosis, whereas its inhibition blocks apoptosis in DP thymocytes.

MEK5 regulates thymocyte apoptosis

To further test the possible roles of MEK5 and ERK5 in thymocyte biology, we set up RTOC with fetal thymocytes from F5 Rag+ TCR transgenic mice and assessed the ability of these T cells to differentiate *in vitro*. T cells expressing the F5 TCR are positively selected on a H-2 D^b background to become CD8$^+$ cells (Mamalaki *et al*, 1993). As shown in Figure 3C, MEK5(A) did not interfere with maturation of the transgenic F5 T cells, as indicated by both the number and representation of the CD8$^+$ SP cells. In these and subsequent experiments, we omit the analyses of MEK5(D)-expressing thymocytes because very few GFP$^+$ cells emerge. We also attempted to induce apoptosis in F5 RTOC system by adding agonist peptide (NP68); however, basting the thymic lobes with peptides failed to induce an apoptotic response, presumably due to the lack of appropriate antigen-presenting cells (Degermann *et al*, 1994). Instead, we treated the re-aggregated thymic lobes with anti-CD3 and anti-CD28 antibodies, which resulted in at least a 10-fold reduction in the percentage of DP cells in the empty virus-infected control, whereas expression of MEK5(A) led to a less than two-fold reduction (Figure 3D). The levels of TCR/CD3 surface expression on MEK5(A)- and empty virus-infected DP cells were similar (Supplementary Figure 2B). As summarized in Figure 3E, the protective effect of MEK5(A) on DP cells was

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also reflected in the absolute cell numbers. Thus, MEK5(A) blocks TCR-mediated apoptosis in thymocytes.

We further evaluated the contribution of the MEK5–ERK5 pathway to thymocyte selection *in vivo*. To this end, we generated bone marrow chimaeras by reconstituting lethally irradiated mice with HSCs that were infected with MEK5(A) or empty virus. We found that the MEK5(D) virus behaved similarly in the stem cell reconstitution system as in the RTOC system in that it significantly inhibited the reconstitution potential of the infected HSCs (data not shown).

To assess the requirement for MEK5 in positive selection, we isolated HSCs from TCR transgenic (Rag+) donors, transduced with empty or MEK5(A) retrovirus, and reconstituted lethally irradiated recipients with positively selecting background. At 5–8 weeks post-transfer, thymocytes from reconstituted mice were analysed for differentiation of transgenic TCR^+ T cells. We found that maturation of transgenic TCR^+ $CD8^+$ SP cells was not consistently affected by the expression of MEK5(A) in three distinct examples of MHC class I-restricted TCR transgenic models (F5, H-Y female, and OT-1, all Rag+, Figure 4A and Supplementary Figure 3B), as judged by the overall representation and the number of transgene $CD8^+$ SP thymocytes (Figure 4C, right panel; PBS-injected F5 mice). Similarly, MEK5(A) did not block the maturation or lineage commitment of $CD4^+$ AND transgenic T cells that were positively selected on the $H\text{-}2^b$ background (Figure 4A). Thus, in transgenic models where T-cell development is accelerated, MEK5 does not have a requisite function in positive selection of either $CD4^+$ or $CD8^+$ SP thymocytes.

The role of the MEK5–ERK5 pathway in negative selection was also assessed in some of these models by injecting with agonist peptide to mimic negatively selecting signals *in vivo*. Injection of F5 TCR transgenic animals with agonist peptide NP68 induces thymocyte deletion with minimal bystander effects (Tarazona *et al*, 1998). As shown in Figure 4B, injection of NP68 peptide into bone marrow chimaeras reconstituted with empty virus-infected stem cells induced a dramatic loss of $V\beta11^+$ DP thymocytes, whereas MEK5(A) expression largely prevented this response (Figure 4B, top panels; average of empty virus control = 9.81 ± 1.77% ($n = 3$); average of MEK5(A) = 42.5 ± 13.54% ($n = 4$); $P < 0.05$). This did not reflect general inhibition of TCR signalling because CD69 upregulation in response to activation was unimpaired by MEK5(A) (Figure 4B, lower panels). In fact, increased numbers of $V\beta11^+$ $CD69^+$ cells remained among the MEK5(A)-expressing population, consistent with the notion that these cells are resistant to activation-induced apoptosis. The

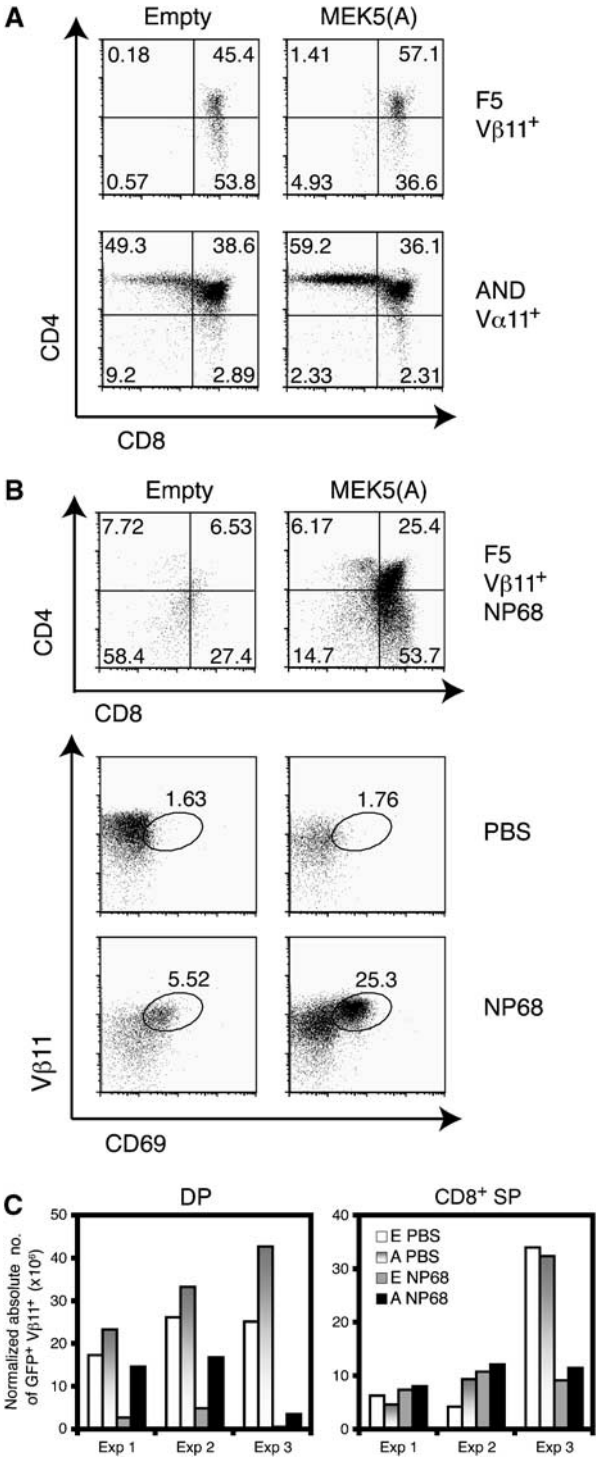

Figure 4 The role of the MEK5–ERK5 pathway in thymocyte selection. (**A**) The role of MEK5 and ERK5 in positive selection was assessed by generating BM chimaeras with empty or MEK5(A) virus-infected stem cells from various TCR transgenic animals, as indicated. Representative CD4/8 profiles gated on GFP^+ transgenic TCR^+ thymocytes 5–8 weeks post-reconstitution are shown. The percentages of cells in individual gates are shown. (**B**) Negative selection was induced by injecting NP68 peptides (50 nmol per animal) into C57BL/6 BM chimaeras reconstituted with retrovirus-infected F5 Rag+ haematopoietic stem cells and analysed 16 h later. Top panels: CD4/8 profiles of cells gated on GFP^+ $V\beta11^+$ (transgenic TCR^+) are shown. Bottom panels: Vβ11/CD69 profiles show that $V\beta11^+$ thymocytes upregulate CD69 activation marker expression in response to NP68 peptide. In both sets, left panels correspond to empty virus control and right panels to MEK5(A) virus-infected cells. (**C**) The protective effect of MEK5(A) on DP thymocytes during peptide-mediated deletion is also indicated by cell numbers. Because of the variability in the level of retroviral infection and chimaerism, normalized numbers of GFP^+ $V\beta11^+$ cells in the DP (left) and $CD8^+$ SP (right) compartments from PBS- or NP68 peptide-injected animals from three independent experiments are shown separately. Because of the variability in the % chimaerism, the absolute cell number of each mouse was normalized to % GFP^+ of a control mouse receiving the empty virus. This was done by taking the absolute cell number of each population multiplied to the ratio of % GFP^+ control/% GFP^+ experimental.

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GFP-negative population in the same animal remained sensitive to peptide-mediated deletion (Supplementary Figure 3C). These results were also supported by the numbers of GFP$^+$ Vβ11$^+$ DP cells, where MEK5(A) expression imparted significant levels of resistance to peptide-induced death in three independent experiments (Figure 4C, left panel). The numbers of CD8$^+$ SP (GFP$^+$ Vβ11$^+$) cells were comparable between the empty virus- and MEK5(A)-expressing populations (Figure 4C, right panel).

The effect of MEK5(A) on DP thymocyte apoptosis was further confirmed by culturing thymocytes from the bone marrow chimaeras in the presence of anti-CD3 and anti-CD28 antibodies. Whereas control DP thymocytes (differentiated from empty virus-infected HSCs) underwent apoptosis with increasing concentrations of anti-CD3 antibody, MEK5(A)-expressing DP thymocytes remained resistant (Figure 5A, left panel). Importantly, MEK5(A) did not inhibit dexamethasone-induced death (Figure 5A, right panel), indicating a specific involvement of MEK5 and ERK5 in TCR-mediated responses. We also evaluated thymocyte apoptosis in the AND transgenic model, in which AND Rag+ TCR$^+$ (Vα11/Vβ3) DP thymocytes undergo apoptosis in the presence of H-2A^s (Vasquez *et al*, 1992). For these experiments, we transferred retrovirus-infected AND (H-2*b*) transgenic bone marrow stem cells into H-2($b \times s$) recipients. Inhibition of apoptosis is expected to increase the number of thymocytes and the percentage of CD4$^+$ SP thymocytes that are positively selected on the A^b MHC class II molecules (Catlett and Hedrick, 2005), although receptor editing has also been shown as a mechanism to escape negative selection in a similar cytochrome *c*-specific MHC class II-restricted 2B4 TCR system (Wang *et al*, 1998). Introduction of MEK5(A) led to a slight augmentation in the percentage (Figure 5B, upper panel; empty virus control = 49.01 ± 4.53% ($n = 8$); MEK5(A) = 64.93 ± 2.92% ($n = 4$); $P < 0.05$) and the number (Figure 5B, lower panel) of GFP$^+$ DP cells, but it did not significantly increase the representation of CD4$^+$ SP thymocytes.

A closer examination of the TCR repertoire, however, revealed a loss of transgenic TCR (Vα11) in thymocytes expressing MEK5(A) while maintaining normal levels of total TCR on the cell surface (Figure 5C, right panel). These results suggest that endogenous TCRα chains have replaced the transgenic TCRα in thymocytes expressing MEK5(A). The TCRα locus is known to rearrange continuously, the extent of which depends on the window of DP cell survival. Increased viability of DP thymocytes in Bcl-x transgenic mice, for example, was shown to lead to an increased frequency of secondary V-to-Jα rearrangements (Guo *et al*, 2002). Thus, replacement of the transgenic TCRα by the endogenous TCRα chains in MEK5(A)-expressing thymocytes is consistent with the notion that MEK5(A) increases the window of survival for DP thymocytes *in vivo*. Importantly, the altered TCR specificity does not permit positive selection of CD4$^+$ SP cells in this model, explaining why the percentage of CD4$^+$ SP cells did not increase.

Finally, the requirement for MEK5 in endogenous superantigen-mediated negative selection was evaluated in BALB/c recipients reconstituted with BALB/c-derived retrovirus-infected HSCs and monitoring the well-documented deletion of Vβ3- and Vβ5-expressing T cells (Kappler *et al*, 1987). Interestingly, MEK5(A) did not block disappearance of the

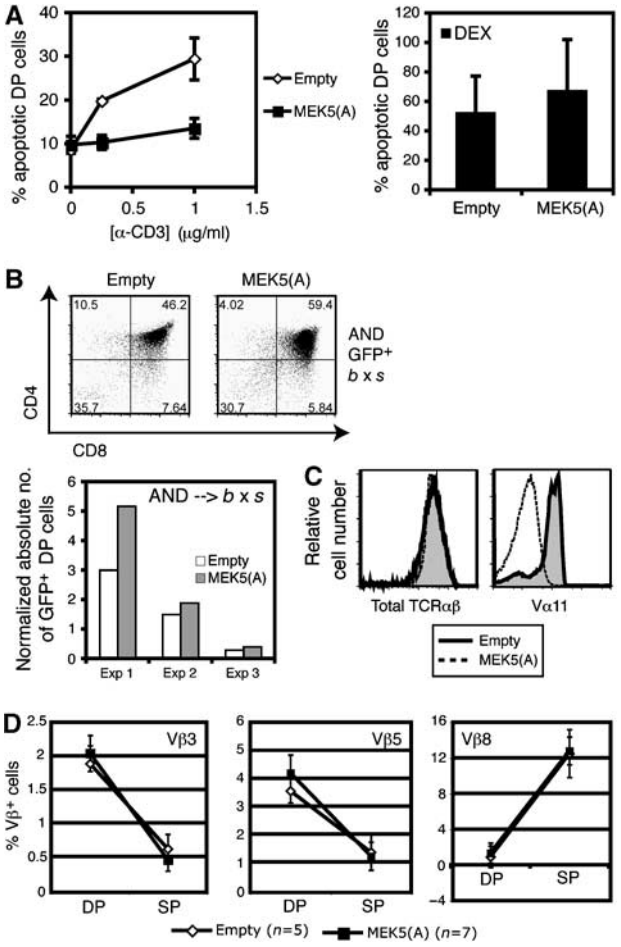

Figure 5 The response of MEK5(A)-expressing thymocytes to apoptotic stimuli in various models. (**A**) *In vitro* deletion of thymocytes from BM chimaeras. Thymocytes from BALB/c chimaeras reconstituted with retrovirus-infected BALB/c bone marrow stem cells were stimulated with plate-bound anti-CD28 (at 1 μg/ml) and varying concentrations of anti-CD3 (left panel) or with dexamethasone at 5 μM (right panel) overnight and analysed by annexin V staining to measure apoptosis. Results representative of three independent experiments are shown (empty control, $n = 5$; MEK5(A), $n = 7$). Error bars indicate standard deviation of triplicate samples (some of the error bars might be too small to be visible). (**B**) Negative selection of AND transgenic thymocytes is assessed in $b \times s$ recipients reconstituted with retrovirus-infected AND Rag+ stem cells. CD4/8 profiles of GFP$^+$ thymocytes transduced by empty or MEK5(A) virus are shown 6 weeks post-transfer (upper). Percentages of cells in the quadrant gates are also shown. Relative numbers of GFP$^+$ DP cells, normalized to percent GFP$^+$, are compared 5–8 weeks post-transfer in three independent experiments (lower). MEK5(A) expression results in a modest but consistent increase in the number of GFP$^+$ DP thymocytes. (**C**) Overlay representation of total TCR and Vα11 profiles for control (solid line with grey fill-in) and MEK5(A) (dotted line) expressing thymocytes are shown to indicate downregulation of Vα11, but not total TCR in MEK5(A)-expressing cells. The profiles are representative of four experiments. (**D**) Endogenous superantigen-induced deletion. Thymocytes from BALB/c BM chimaeras reconstituted with retrovirus-infected BALB/c haematopoietic stem cells were analysed by staining for CD4, CD8, and Vβ3, Vβ5, or Vβ8. Percentages of Vβ$^+$ cells in the DP and SP (CD4$^+$ SP) populations are shown to compare the efficiency of deletion during the DP-to-SP transition. Vβ8 is included as a non-deleting control. The error bars indicate standard deviation of the mean.

Vβ3- and Vβ5-expressing thymocytes during the DP-to-SP transition (Figure 5D), indicating that MEK5–ERK5 is not required for superantigen-induced cell death. Nonetheless,

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the increased survival time of MEK5(A)-expressing DP cells as evidenced by the complete replacement of the AND transgenic Vα by the endogenous Vα proteins, together with the results from the F5 peptide-mediated deletion *in vivo* and anti-CD3/CD28-mediated deletion *in vitro*, indicates that the MEK5–ERK5 axis constitutes a major pathway in various models of thymocyte cell death. On the other hand, MEK5's role in positive selection is minor and may involve indirect mechanisms, being influenced by the nature of the particular TCR expressed on the cells undergoing selection.

Discussion

ERK5 represents a novel class of MAPK that exerts an effect as a potent transcriptional co-regulator. Its catalytic domain is most closely related to ERK1/2, but it shares target specificity with p38 in being able to activate MEF2-dependent transcription. Our study indicates that the MEK5–ERK5 pathway has an important function in thymocyte apoptosis while having a minor function in positive selection. Blocking ERK5 (through expression of a catalytically inactive MEK5 mutant), p38, or JNK pathway exerts a significant impact on *in vitro* deletion by anti-CD3/CD28 (this paper; Sugawara *et al*, 1998; Sabapathy *et al*, 2001). Dominant-negative MEK5 protein also inhibits apoptotic signals induced by agonist peptide *in vivo* but not negative selection induced by superantigen. Inhibition of the MEK5–ERK5 pathway in the negatively selecting AND model (MHC class II restricted) caused an accumulation of cells that have lost transgenic TCRα. As total TCR expression on the cell surface remains high, these receptors represent those with alternate endogenous α chains that have been generated through the receptor editing process. Receptor editing has been shown to be a mechanism utilized by developing T cells to escape negative selection in a number of TCR transgenic models (McGargill *et al*, 2000), including a cytochrome *c*-specific 2B4 model (Wang *et al*, 1998) similar to the AND model used in our study. Increased viability of DP thymocytes has also been correlated with increased frequency of secondary V-to-Jα rearrangements (Guo *et al*, 2002). Thus, accumulation of a large number of cells that have replaced the transgenic TCRα with the endogenous TCRα chains in MEK5(A) AND bone marrow chimaeras is consistent with the notion that MEK5(A) increases the window of survival for DP thymocytes *in vivo*. In addition, as a consequence of altered TCR specificity, positive selection of transgenic TCR^+ thymocytes becomes less efficient, and the DP-to-CD4 SP transition is inhibited in this system. In contrast, continuous rearrangement of the TCRα chain did not affect F5 Rag+ transgenic TCR model, as these cells retained their ability to respond to cognate peptide ligand *in vivo* (thymocytes upregulated CD69 following peptide challenge). The differences in the outcome between F5 and AND transgenic models might reflect that apoptosis in response to class I-, but not class II-, mediated signal is sensitive to inhibition of the MEK5–ERK5 pathway, perhaps due to weaker TCR signals triggered by a lower level recruitment of Lck when class I MHC is engaged. However, it is more likely that the difference reflects the ability of cytochrome *c*-specific TCR to undergo receptor editing to avoid negative selection (Wang *et al*, 1998) and the inability of F5 TCR to do so. The latter is similar to the transgenic H-Y TCR, which has been shown to be resistant to the receptor editing process (Buch *et al*, 2002; Holman *et al*, 2003).

Gene profile analyses in models with defects in negative selection have pointed to at least two effector pathways of apoptosis during negative selection: Nur77 and Bim (Liston *et al*, 2004; Zucchelli *et al*, 2005). Nur77 and its homologues represent an orphan steroid receptor family induced as immediate-early genes following TCR engagement. Nur77 may command apoptosis through multiple signalling cascades, employing both transcription-dependent and -independent pathways. Nur77 increases expression of FasL, TRAIL, and NDG1, a novel apoptotic gene, in thymocytes from Nur77 transgenic mice (Rajpal *et al*, 2003). Nur77 also has been shown to interact with normally antiapoptotic Bcl-2 in mitochondria and convert it to a pro-apoptotic form in thymocytes (Thompson and Winoto, 2008). Deficiency of Nur77 alone in thymocytes does not impair negative selection, indicating other members of the Nur77 family may compensate in its absence (for review, see Sohn *et al*, 2003). Nur77 and related proteins can bind to the same DNA element and thus share target specificity. Indeed, a dominant-negative form of Nor-1/Nur77 can block, and conversely, overexpression of Nor-1 can induce, apoptosis of thymocytes. Thus, the ability of ERK5 to co-regulate expression of Nur77 and Nor-1 supports its role as a co-activator for MEF2-driven transcription of this family of apoptotic genes during T-cell development. Bim is a BH3-only member of the Bcl-2 family proteins that triggers the mitochondrial apoptotic pathway. Bim-deficient thymocytes exhibit significant defects in their ability to undergo death (Bouillet *et al*, 2002), and thymocytes from autoimmune-prone strains of mice express reduced levels of Bim (Liston *et al*, 2004; Zucchelli *et al*, 2005). Interestingly, activation of the ERK1/2 pathway has been shown to activate Bim ubiquitination (Akiyama *et al*, 2003). If this also occurs in T cells, it might represent one possible mechanism for positively selected thymocytes to bypass Bim-mediated apoptosis.

Nur77 and Bim may differentially regulate various forms of thymocyte apoptosis. For example, expression of Nur77 dominant-negative proteins fails to affect superantigen-mediated cell death, whereas Bim deficiency does (Calnan *et al*, 1995; Bouillet *et al*, 2002). This may reflect specific requirements for triggering Bim versus Nur77 induction *in vivo*, pertaining to the spatial constraints of negatively selecting ligands or co-stimulatory molecules. Thus, heterogeneity in both the cellular makeup and the antigenic repertoire representing 'self' may contribute towards generating distinct MAPK signals in thymocytes undergoing apoptosis. In such a model, ERK5, p38, and JNK may each preferentially respond to different types of stimuli to induce apoptosis in immature thymocytes. As a result, their combined responses ensure elimination of T cells that may react inappropriately.

With the report on ERK1 and ERK2 conditional knockout models, it now appears indisputable that ERK1 and ERK2 have overlapping but requisite functions in positive selection (Fischer *et al*, 2005). Importantly, the block in positive selection is virtually complete in mice lacking both ERK1 and ERK2, making it difficult to argue for existence of additional pathways that regulate positive selection. In this respect, it is interesting that inhibition of the MEK5–ERK5 pathway correlates with a slight decrease in representation of the $CD4^+$ SP thymocytes expressing heterogeneous TCRs

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(in both C57BL/6 and BALB/c bone marrow reconstitution models). As MEK5(A) did not impair maturation of SP cells in the majority of transgenic models we tested, ERK5 is not absolutely required for positive selection. However, it is possible that ERK5 influences positive selection of $CD4^+$ SP cells representing normal endogenous repertoire but is ineffective in transgenic models wherein T-cell development is accelerated. Alternatively, we must consider the possibility that ERK5 has additional functions in T-cell homoeostasis following selection and that this is reflected in the partial reduction in the percentage of $CD4^+$ SP cells. For example, ERK5 may be required to enhance survival and/or maintenance of mature T cells. In this regard, the fact that ERK5 is activated in response to IL-7 (Sohn *et al*, 2005), a known survival-promoting cytokine, and that it induces LKLF (a survival and quiescence factor; Kuo *et al*, 1997), supports the potential involvement of this unique MAPK in mature T-cell homoeostasis post-selection.

The dichotomy of cell-fate decisions made through the MAPK pathways further begs the question of how different MAPKs commit cells to survival versus death at the molecular level. Biochemical activation profiles of ERK1/2, ERK5, and p38 offer an insight into the differential involvement of these kinases during thymocyte selection. Previous reports (Werlen *et al*, 2000; Mariathasan *et al*, 2001; McNeil *et al*, 2005; Daniels *et al*, 2006) comparing MAPK activation profiles have suggested that positively selecting weak agonist (e.g. EIINFEKL) induced weak but sustained/biphasic ERK1/2 response, whereas negatively selecting strong agonist (e.g. SIINFEKL) induced strong and transient ERK1/2 response when stimulated with the same concentrations of peptides. p38 and JNK, in contrast, were activated by both types of ligands with similar intensities and kinetics (Werlen *et al*, 2000). Given that ERK5 shared structural similarity with ERK1/2 but target specificity with p38, we were interested in determining whether ERK5 behaved more like ERK1/2 or p38 in thymocytes poised to undergo selection. Our results demonstrated that ERK5 responded rapidly like ERK1/2, but like p38, responded to both strong and weak agonists with similar intensity and kinetics. Furthermore, ERK1/2 appears able to 'qualitatively' differentiate negatively and positively selecting ligands (under conditions that induce 'quantitatively equivalent' TCR signals), whereas p38 and JNK, and now ERK5, lack such sensitivity. Together with our *in vivo* data, these data support a model wherein ERK1/2, by virtue of its ability to respond differently to strong versus weak agonists, has a major function in positive selection, whereas ERK5, p38, and JNK, which are rather insensitive to the 'qualitative' difference between positively and negatively selecting signals, have more prominent (and potentially overlapping) functions in negative selection. How these 'qualitative' and 'quantitative' differences translate into intracellular molecular events remains unclear. Additional studies will be necessary to dissect out all the molecular pathways of TCR-mediated apoptosis during T-cell development.

Materials and methods

Thymocyte stimulation and analyses of MAPK activation

Thymocytes from MHC-deficient mice were pre-incubated at 37°C for 2 h, then stimulated with anti-CD3 ascites (500A2) with anti-CD28 ascites at 1:1000 for indicated times. Activation by peptide stimulation was done following a previously described protocol (Werlen *et al*, 2000), using peptide-coated, glutaraldehyde-fixed EL4 cells as antigen-presenting cells. For immunoblotting, whole-cell lysates were prepared by disrupting the cells in a buffer containing 1% Triton X-100, resolved in SDS–PAGE, transferred onto nitrocellulose membrane, and blotted with various anti-phospho-MAPK antibodies. All except anti-ERK5 antibody (Kasler *et al*, 2000) were purchased from commercial sources: phospho-ERK5 Ab (Biosource), phospho-ERK1/2 (Santa Cruz), and phospho-p38 (Cell Signaling).

Retroviral transduction of DO11.10 hybridoma cells and Amaxa transfection of primary thymocytes

Production of retrovirus and infection of DO11.10 cells were performed following a previously published protocol (Pear *et al*, 1993), with a modification that 293T cells were co-transfected with retroviral construct and pCL-ECO packaging construct, instead of using standard packaging cell lines. At 1 week after infection, infected cells were stimulated with ionomycin at 0.5 μM and PMA at 2.5 ng/ml before analysis.

For Amaxa transfection of primary thymocytes, total thymocytes from 6- to 8-week-old BALB/c mice were harvested and transfected with pCI (empty control) or pCI-MEK5(D) as per the manufacturer's instruction. At 22 h post-transfection, unsorted cells were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies for 4 h before RNA isolation.

Quantitative RT–PCR

The relative abundance of transcripts for *nur77*, *nor-1*, *bim*, *lklf*, and *egr-1* was measured by SYBR green analyses on ABI 5700 instrument using absolute quantification protocol. Total RNA was isolated from T cells using Qiagen RNeasy kit. Following DNase treatment, first-strand synthesis was performed by reverse transcription using oligo$(dT)_{15}$ primer and SuperScriptII (Invitrogen). Relative abundance was determined by normalization against house-keeping genes (HPRT or γ-actin). The sequences for the primers are as follows. *nur77* for 5′-TCCTCATCACTGATCGACACG; *nur77* rev 5′-AGCTCTTCCACCCGACGAG; *nor-1* for 5′-GTCCACTTAC GCCACGCAG; *nor-1* rev 5′-TGATCCCCGTGCTACCGA; *bim* for 5′-GC CAAGCAACCTTCTGATGTAA; *bim* rev 5′-TTGTCCACCTTCTCTGTC ACACTC; *egr-1* for 5′-CTCCGACCTCTTCATCCTCG; *egr-1* rev 5′-AGG AAGACGATGAAGCAGCTG; *hprt* for 5′-GTTGGATACAGGCCAGACT TTGTTG; and *hprt* rev 5′-GAGGGTAGGCTGGCCTATAGGCT. The primer sequences for *lklf* and *γ-actin* have previously been published (Sohn *et al*, 2005).

Flow cytometry

Apoptosis was measured by annexin V staining (BD Pharmingen) of live-gated GFP^+ population. Differentiation of thymocytes was assessed by analyses of live cell-gated, GFP^+ population for developmental marker expression: PE-Cy5-anti-CD4 (eBioscience), PE-Texas red-anti-CD8α (Caltag), biotinylated anti-Vβ11 (BD Pharmingen), PE-anti-Vα11 (BD Pharmingen), and PE-anti-CD69 (Caltag), biotinylated anti-Vβ3, anti-Vβ5, and anti-Vβ8 (BD Pharmingen), and PE-anti-TCRβ (H57-597; eBioscience).

In vivo peptide-mediated deletion

C57BL/6 recipients reconstituted with F5 Rag+ HSCs were injected intraperitoneally with either PBS alone or with NP68 peptide solution in PBS (50 nmol per animal) in 200 μl volume as per standard protocol, at 5–8 weeks post-transfer.

In vitro deletion

Thymocytes from bone marrow chimaeras were cultured overnight in wells pre-coated with anti-CD3 (500A2) and anti-CD28, at indicated concentrations, or with dexamethasone at 5 μM. Apoptosis was measured by staining with annexin V and analysing by flow cytometry on GFP^+ DP-gated populations.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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References

Akiyama T, Bouillet P, Miyazaki T, Kadono Y, Chikuda H, Chung UI, Fukuda A, Hikita A, Seto H, Okada T, Inaba T, Sanjay A, Baron R, Kawaguchi H, Oda H, Nakamura K, Strasser A, Tanaka S (2003) Regulation of osteoclast apoptosis by ubiquitylation of proapoptotic BH3-only Bcl-2 family member Bim. *EMBO J* **22:** 6653–6664

Alberola-Ila J, Hogquist KA, Swan KA, Bevan MJ, Perlmutter RM (1996) Positive and negative selection invoke distinct signaling pathways. *J Exp Med* **184:** 9–18

Bouillet P, Purton JF, Godfrey DI, Zhang LC, Coultas L, Puthalakath H, Pellegrini M, Cory S, Adams JM, Strasser A (2002) BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. *Nature* **415:** 922–926

Buch T, Rieux-Laucat F, Forster I, Rajewsky K (2002) Failure of HY-specific thymocytes to escape negative selection by receptor editing. *Immunity* **16:** 707–718

Calnan BJ, Szychowski S, Chan FK, Cado D, Winoto A (1995) A role for the orphan steroid receptor Nur77 in apoptosis accompanying antigen-induced negative selection. *Immunity* **3:** 273–282

Catlett IM, Hedrick SM (2005) Suppressor of cytokine signaling 1 is required for the differentiation of CD4+ T cells. *Nat Immunol* **6:** 715–721

Daniels MA, Teixeiro E, Gill J, Hausmann B, Roubaty D, Holmberg K, Werlen G, Hollander GA, Gascoigne NR, Palmer E (2006) Thymic selection threshold defined by compartmentalization of Ras/MAPK signalling. *Nature* **444:** 724–729

Degermann S, Surh CD, Glimcher LH, Sprent J, Lo D (1994) B7 expression on thymic medullary epithelium correlates with epithelium-mediated deletion of V beta 5+ thymocytes. *J Immunol* **152:** 3254–3263

Delgado P, Fernandez E, Dave V, Kappes D, Alarcon B (2000) CD3 delta couples T-cell receptor signalling to ERK activation and thymocyte positive selection. *Nature* **406:** 426–430

Fischer AM, Katayama CD, Pages G, Pouyssegur J, Hedrick SM (2005) The role of Erk1 and Erk2 in multiple stages of T cell development. *Immunity* **23:** 431–443

Guo J, Hawwari A, Li H, Sun Z, Mahanta SK, Littman DR, Krangel MS, He YW (2002) Regulation of the TCRalpha repertoire by the survival window of CD4(+)CD8(+) thymocytes. *Nat Immunol* **3:** 469–476

Hayashi M, Kim SW, Imanaka-Yoshida K, Yoshida T, Abel ED, Eliceiri B, Yang Y, Ulevitch RJ, Lee JD (2004) Targeted deletion of BMK1/ERK5 in adult mice perturbs vascular integrity and leads to endothelial failure. *J Clin Invest* **113:** 1138–1148

Hernandez-Hoyos G, Anderson MK, Wang C, Rothenberg EV, Alberola-Ila J (2003) GATA-3 expression is controlled by TCR signals and regulates CD4/CD8 differentiation. *Immunity* **19:** 83–94

Hogquist KA, Jameson SC, Heath WR, Howard JL, Bevan MJ, Carbone FR (1994) T cell receptor antagonist peptides induce positive selection. *Cell* **76:** 17–27

Holman PO, Walsh ER, Hogquist KA (2003) The central tolerance response to male antigen in normal mice is deletion and not receptor editing. *J Immunol* **171:** 4048–4053

Kappler JW, Roehm N, Marrack P (1987) T cell tolerance by clonal elimination in the thymus. *Cell* **49:** 273–280

Kasler H, Victoria J, Duramad O, Winoto A (2000) ERK5 is a novel type of mitogen-activated protein kinase containing a transcriptional activation domain. *Mol Cell Biol* **20:** 8382–8389

Kato Y, Kravchenko VV, Tapping RI, Han J, Ulevitch RJ, Lee J-D (1997) BMK1/ERK5 regulates serum-induced early gene expression through transcription factor MEF2C. *EMBO J* **16:** 7054–7066

Kato Y, Tapping RI, Huang S, Watson MH, Ulevitch RJ, Lee JD (1998) Bmk1/Erk5 is required for cell proliferation induced by epidermal growth factor. *Nature* **395:** 713–716

Kuo CT, Veselits ML, Leiden JM (1997) LKLF: a transcriptional regulator of single-positive T cell quiescence and survival. *Science* **277:** 1986–1990

Liston A, Lesage S, Gray DH, O'Reilly LA, Strasser A, Fahrer AM, Boyd RL, Wilson J, Baxter AG, Gallo EM, Crabtree GR, Peng K, Wilson SR, Goodnow CC (2004) Generalized resistance to thymic deletion in the NOD mouse; a polygenic trait characterized by defective induction of Bim. *Immunity* **21:** 817–830

Mamalaki C, Elliot T, Norton T, Yannoutsos N, Townsend AR, Chandler P, Simpson E, Kioussis D (1993) Positive and negative selection in transgenic mice expressing a T-cell receptor specific for influenza nucleoprotein and endogenous superantigen. *Dev Immunol* **3:** 159–174

Mariathasan S, Ho SS, Zakarian A, Ohashi PS (2000) Degree of ERK activation influences both positive and negative thymocyte selection. *Eur J Immunol* **30:** 1060–1068

Mariathasan S, Zakarian A, Bouchard D, Michie AM, Zuniga-Pflucker JC, Ohashi PS (2001) Duration and strength of extracellular signal-regulated kinase signals are altered during positive versus negative thymocyte selection. *J Immunol* **167:** 4966–4973

McGargill MA, Derbinski JM, Hogquist KA (2000) Receptor editing in developing T cells. *Nat Immunol* **1:** 336–341

McNeil LK, Starr TK, Hogquist KA (2005) A requirement for sustained ERK signaling during thymocyte positive selection *in vivo*. *Proc Natl Acad Sci USA* **102:** 13574–13579

Mody N, Leitch J, Armstrong C, Dixon J, Cohen P (2001) Effects of MAP kinase cascade inhibitors on the MKK5/ERK5 pathway. *FEBS Lett* **502:** 21–24

Pear WS, Nolan GP, Scott ML, Baltimore D (1993) Production of high-titer helper-free retroviruses by transient transfection. *Proc Natl Acad Sci USA* **90:** 8392–8396

Rajpal A, Cho YA, Yelent B, Koza-Taylor PH, Li D, Chen E, Whang M, Kang C, Turi TG, Winoto A (2003) Transcriptional activation of known and novel apoptotic pathways by Nur77 orphan steroid receptor. *EMBO J* **22:** 6526–6536

Regan CP, Li W, Boucher DM, Spatz S, Su MS, Kuida K (2002) Erk5 null mice display multiple extraembryonic vascular and embryonic cardiovascular defects. *Proc Natl Acad Sci USA* **99:** 9248–9253

Sabapathy K, Kallunki T, David JP, Graef I, Karin M, Wagner EF (2001) c-Jun NH2-terminal kinase (JNK)1 and JNK2 have similar and stage-dependent roles in regulating T cell apoptosis and proliferation. *J Exp Med* **193:** 317–328

Sohn SJ, Li D, Lee LK, Winoto A (2005) Transcriptional regulation of tissue-specific genes by the ERK5 mitogen-activated protein kinase. *Mol Cell Biol* **25:** 8553–8566

Sohn SJ, Rajpal A, Winoto A (2003) Apoptosis during lymphoid development. *Curr Opin Immunol* **15:** 209–216

Sohn SJ, Sarvis BK, Cado D, Winoto A (2002) ERK5 MAPK regulates embryonic angiogenesis and acts as a hypoxia-sensitive repressor of vascular endothelial growth factor expression. *J Biol Chem* **277:** 43344–43351

Starr TK, Jameson SC, Hogquist KA (2003) Positive and negative selection of T cells. *Annu Rev Immunol* **21:** 139–176

Sugawara T, Moriguchi T, Nishida E, Takahama Y (1998) Differential roles of ERK and p38 MAP kinase pathways in positive and negative selection of T lymphocytes. *Immunity* **9:** 565–574

Tarazona R, Williams O, Moskophidis D, Smyth LA, Tanaka Y, Murdjeva M, Wack A, Mamalaki C, Kioussis D (1998) Susceptibility and resistance to antigen-induced apoptosis in the thymus of transgenic mice. *J Immunol* **160:** 5397–5403

Thompson J, Winoto A (2008) During negative selection, Nur77 family proteins translocate to mitochondria where they associate with Bcl-2 and expose its proapoptotic BH3 domain. *J Exp Med* **205:** 1029–1036

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Vasquez N, Kaye J, Hedrick SM (1992) *In vivo* and *in vitro* clonal deletion of double-positive thymocytes. *J Exp Med* **175:** 1307–1316

Wang F, Huang CY, Kanagawa O (1998) Rapid deletion of rearranged T cell antigen receptor (TCR) Valpha-Jalpha segment by secondary rearrangement in the thymus: role of continuous rearrangement of TCR alpha chain gene and positive selection in the T cell repertoire formation. *Proc Natl Acad Sci USA* **95:** 11834–11839

Wang X, Merritt AJ, Seyfried J, Guo C, Papadakis ES, Finegan KG, Kayahara M, Dixon J, Boot-Handford RP, Cartwright EJ, Mayer U, Tournier C (2005) Targeted deletion of mek5 causes early embryonic death and defects in the extracellular signal-regulated kinase 5/myocyte enhancer factor 2 cell survival pathway. *Mol Cell Biol* **25:** 336–345

Werlen G, Hausmann B, Palmer E (2000) A motif in the alpha beta T-cell receptor controls positive selection by modulating ERK activity. *Nature* **406:** 422–426

Winoto A, Littman DR (2002) Nuclear hormone receptors in T lymphocytes. *Cell* **109:** S57–S66

Yan L, Carr J, Ashby PR, Murry-Tait V, Thompson C, Arthur JS (2003) Knockout of ERK5 causes multiple defects in placental and embryonic development. *BMC Dev Biol* **3:** 11

Zucchelli S, Holler P, Yamagata T, Roy M, Benoist C, Mathis D (2005) Defective central tolerance induction in NOD mice: genomics and genetics. *Immunity* **22:** 385–396

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