Positive and Negative Selection Invoke Distinct Signaling Pathways

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Summary

During T cell development, interaction of the T cell receptor (TCR) with cognate ligands in the thymus may result in either maturation (positive selection) or death (negative selection). The intracellular pathways that control these opposed outcomes are not well characterized. We have generated mice expressing dominant-negative Ras (dnRas) and Mek-1 (dMek) transgenes simultaneously, either in otherwise normal animals, or in animals expressing a transgenic TCR, thereby permitting a comprehensive analysis of peptide-specific selection. In this system, thymocyte maturation beyond the $CD4^+8^+$ stage is blocked almost completely, whereas negative selection, assessed using an in vitro deletion protocol, is quantitatively intact. This suggests that activation of the mitogen-activated protein kinase (MAPK) cascade is necessary for positive selection, but irrelevant for negative selection. Generation of γ/δ and of $CD4^-8^-$ α/β T cells proceeds normally despite blockade of the MAPK cascade. Hence, only cells that mature via conventional, TCR-mediated repertoire selection require activation of the MAPK pathway to complete their maturation.

Expansion of immature progenitor cells intrathymically gives rise to three distinct types of T lymphocytes: conventional CD4- or CD8-bearing T cells that utilize α/β-type TCRs, $\alpha\beta$ cells lacking CD4 and CD8 (and generally expressing the NK1.1 marker), and T cells expressing the γ/δ TCR. In each cell lineage, development proceeds through a series of defined stages to ensure that each lymphocyte expresses a functional TCR. In the most thoroughly studied case, $CD4^+8^+$ (double-positive) thymocytes that express $\alpha\beta$ TCRs interact with stromal cells that display class I and II products of the MHC. Most double-positive thymocytes bear TCRs that do not recognize the MHC molecules present in the thymus, and these cells die relatively rapidly through a process termed "death by neglect" (1). In contrast, recognition of self-MHC antigen-presenting structures, presumably bearing bound peptide, can trigger either functional differentiation (positive selection) or apoptosis (negative selection).

This paradoxical feature of T cell development, that a single receptor can deliver signals to induce either maturation or death, is central to the process of thymocyte development, but remains poorly understood. A favored hypothesis (the avidity model) postulates that positively selecting peptide/MHC complexes interact with the TCR with low affinity, or are present in low numbers, and deliver a weak signal that rescues cells from their default fate (death by neglect) without inducing apoptosis. In contrast,

an especially avid interaction between individual TCR-bearing thymocytes and antigen-presenting stromal cells could provide a strong signal, thereby provoking deletion (2).

Experiments in which peptide antigens are added at increasing concentrations to cultured fetal thymuses provide some support for this model in that antigen dose was found to regulate the yield of mature-appearing $CD4^-8^+$ cells (3, 4). However it is not clear that cells derived via this protocol have truly acquired mature, functional characteristics. Other experiments (5) suggest that agonist peptides invariably provoke negative selection, whereas true positive selection, that yielding functional cells, is only achieved using nonstimulatory peptide ligands (those with antagonist properties) in thymic organ culture (5, 6). These observations argue that the TCR-coupled signaling mechanism in developing thymocytes responds in a qualitatively different way to distinct peptide/MHC complexes: it can sense the character of an agonist peptide, even when it is present in very low abundance, and thereafter deliver an apoptotic signal.

If the responses to positively and negatively selecting peptides differ qualitatively, then the T cell signaling mechanisms stimulated by these two classes of ligands should also be distinguishable. Some information regarding the importance of different TCR-coupled signaling molecules in selection has emerged through study of animals bearing targeted mutations in the genes that encode them. For

example, both positive and negative selection of CD4⁺8⁺ precursors proceed unimpaired in *fyn* null animals (7, 8). In contrast, thymocytes lacking the zeta-associated protein 70 (ZAP-70)[1] kinase cannot sustain either positive or negative selection (9). Hence, the differential recruitment of signaling complexes that permits a distinction between positively and negatively selecting ligands must occur at a point distal (in biochemical terms) to ZAP-70 in the signal transduction cascade.

In previous studies, we demonstrated that inhibition of signaling through p21^{ras} or the mitogen-activated protein kinase (MAPK) Mek1 (achieved in both cases by thymocyte-specific expression of a dominant-negative transgene) caused a marked reduction in the efficiency of positive selection without affecting negative selection (8, 10). These observations suggested that the mitogen-activated protein kinase (MAPK) pathway might be involved specifically in positive selection.

Although expression of high levels of dominant-negative Ras (dnRas) or Mek-1 (dMek) proteins in thymocytes substantially blocks thymocyte maturation, this inhibition rarely exceeds 70%. Moreover, the inhibition of MAPK activation, while proportional to the amount of dMek transgene expressed in the thymocytes, was never complete. Hence, it remained possible that quantitative differences in the strength of TCR-derived signals could explain the relatively discrete interdiction of positive selection in these transgenic mice. If, using the same signal transduction components, negatively selecting stimuli deliver substantially stronger signals than do positively selecting stimuli, partial inhibition of the MAPK cascade might attenuate only the outcomes from the weakest signaling events. This argument predicts that complete inhibition of Ras signaling would affect both positive and negative selection, just as is seen with targeted disruption of the *Zap-70* gene.

To address this possibility, we have imposed a more profound block in the MAPK cascade by simultaneously expressing both dnRas and dMek-1, and have examined the efficiency of negative selection in such cells using an in vitro deletion system where the strength of the signal can be controlled experimentally. We have also studied the development of γδ and NK1.1⁺αβ T cells in these mice. Our results demonstrate that stimulation of the MAPK cascade is absolutely required for the satisfactory selection of mature TCR-α/β–bearing single-positive cells, but is apparently irrelevant for other thymocyte lineages. Moreover, negative selection proceeds unimpaired, and with identical dose–response characteristics, in the absence of effective Ras pathway signaling. These results indicate that TCRs can deliver very different types of signals by variably recruiting distinct, downstream signal transduction pathways.

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[1] *Abbreviations used in this paper:* dMek, dominant-negative Mek-1; dnRas, dominant-negative Ras; HSA, heat-stable antigen; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; ZAP-70, zeta-associated protein 70.

Materials and Methods

Transgenic Mice. The generation of dnRas (lines 15465 and 15728), dMek (line A1665), OT-I and H-Y TCR transgenic mice has been previously described (5, 8, 10, 11).

Peptides. Peptides were synthesized at the Biopolymer Synthesis Facility, Department of Immunology (University of Washington) using an Applied Biosystems (Foster City, CA) instrument.

Flow Cytometric Analysis. Thymocytes were stained with saturating concentrations of antibody at 4°C for 30 min. Cells were examined for surface expression of the following molecules: CD4 (PE L3T4), CD8 (FITC Ly-2; Caltag Laboratories, South San Francisco, CA); CD3ε (FITC- PE- or biotin-conjugated, 145-2C11), HSA (biotinylated J11d), αβ TCR (biotinylated H57-597), γδ TCR (PE-CGL3), Vγ3 (FITC-563), and CD69 (biotinylated H1.2FC; PharMingen, San Diego, CA). The H-Y TCR α chain was stained with biotinylated T3.70 (11), and the OVA-TCR with the Vα2-specific antibody B20.1 (12). Biotinylated antibodies were visualized using trichrome-labeled streptavidin (Caltag). Analyses were performed using a FACScan® flow cytometer with Lysis II software (Becton Dickinson & Co., San Jose, CA). Subsequent data analysis used ReproMan software (True Facts Software, Seattle, WA). Cell sorting was performed on a dual laser FACStar Plus® instrument (Becton Dickinson & Co.).

Western Blotting. Thymocytes were lysed on ice for 15 min with lysis buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 5 mM EDTA, 50 mM NaF, 0.4 mM Na$_3$VO$_4$, 10 mM iodoacetamide, 5 mM sodium pyrophosphate, 1 mM PMSF, and 1 mg/ml aprotinin, leupeptin, pepstatin A, and chymostatin) and then centrifuged for 5 min at 15,000 g. The protein concentration in the supernatants was determined by the Bradford method. The lysates were fractionated by 10% SDS-PAGE gels under reducing conditions and then electrotransferred to nitrocellulose paper. The nitrocellulose filters were blocked for 45 min at 37°C in PBS, pH 7.2, 0.1% Tween-20, and 5% milk powder, and then incubated with primary antibody (ras 10, Ab-3 from Oncogene Sciences, Uniondale, NY; and anti-Mek mAb from Transduction Laboratories, Lexington, KY) for 1 h. After three 5-min washes with PBS/01% Tween-20, the blots were incubated with horseradish peroxidase–conjugated sheep anti–mouse antiserum for 30 min, washed extensively, and developed by enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL), according to the manufacturer's instructions.

Suspension Deletion Assay. EL4 cells were irradiated and plated at 5 × 10^4/well in round bottom sterile 96-well plates. The different peptides, OVAp (SIINFEKL), E1 (EIINFEKL), V-OVA (RGYNYEKL), and p-SER (SSYSYSSL) were diluted in RPMI at different concentrations and added to the plates. Total thymocytes from OT-I, OT-I/dnRas, OT-I/dMek, and OT-I/dnRas/ dMek were then added to the plates at 5 × 10^5/well and incubated for 18 h at 37°C. An aliquot of the thymocytes was stained at time 0. At the end of the culture, the cells were stained with biotinylated CD69, anti-CD4-PE, anti-CD8-FITC, and streptavidin-Tricolor. The number of viable double positive cells was determined by means of a live gate and a tight DP gate. EL4 cells were excluded from the analysis based on forward and right-angle light scattering. Induction of CD69 was determined by recording the mean fluorescence intensity of FL3 on total double positive thymocytes.

PCR. Genomic DNA was prepared as described by Laird et al. (13), and PCR amplification reactions were performed in 50 μl reaction buffer (50 mM KCl, 20 mM Tris-HCl, pH8.3, 2.5 mM MgCl$_2$, and 0.1 mg/ml BSA) containing 0.5 μg template DNA,

1 mM primers, 0.2 mM each dNTP (Pharmacia, Piscataway, NJ), and 1 U AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Foster City, CA). The amplification cycle (1 min at 95°C, 2 min at 60°C, and 5 min at 72°C) was repeated 25 times. The 5' primers were specific for H-Ras (5'-CCAAGTCCTTTGAAGACATC-CATCAG-3') and Mek-1 (5'-CCCGGGGGATCCCTTGCT-CATACGGA-3') and the 3' for the hHGH sequence present in the transgene (5'- TGGCAACTTCCAAGGCCAGGAGAG-3').

Results

The combination of dnRAS and dMek Completely Abrogates Generation of Single-positive αβ T Cells. To generate mice in which activation of the Ras signaling pathway in thymocytes was severely impaired, we crossed animals from the transgenic lines 15465 (dnRas) and A1665 (dMek) and examined the development of T cells in their progeny (see Materials and Methods). Doubly transgenic thymocytes expressed the same amounts of each transgene-encoded protein as did cells from the parental lines (data not shown). Although the thymuses of the doubly transgenic animals were normal in size and cellularity, phenotypic analysis of the thymocytes revealed a dramatic decrease in the generation of single-positive, TCR^{hi} cells. Fig. 1 demonstrates that the proportion of single-positive thymocytes, reduced

to ~40% of normal in the singly transgenic animals, was further reduced in the doubly transgenic mice to <10% of control values. Typically, the number of CD4$^+$8$^-$ or CD4$^-$8$^+$ cells was reduced to <1% of total thymocytes, and the proportion of CD3^{hi} cells, normally ≈15%, was reduced to ≈1%, most of which represented products of different lineages (Fig. 1 and see below). Hence, the two transgenes, acting in concert, produce a much more complete blockade of thymocyte development than does either alone.

It is worth noting that the yield of mature cells in the doubly transgenic mice corresponds to what would be expected if the inhibitory effects of one transgene were imposed on those cells that escape the influence of the other. For example, the yield of mature cells relative to those in normal animals is ≈0.4 in mice expressing dnRas or dMek, and (0.4 × 0.4) = 0.016 in the case where both transgenes were present. This result is consistent with biochemical and genetic data derived from other systems that position Mek-1 downstream of p21^{ras} in a linear signaling cascade (14–16).

Distinguishing Positive and Negative Selection in the H-YTCR System. Since the appearance of single-positive cells is almost completely blocked in mice expressing both dnRas and dMek transgenes, we tested to see whether TCR signals that stimulate negative selection were in any way dis-
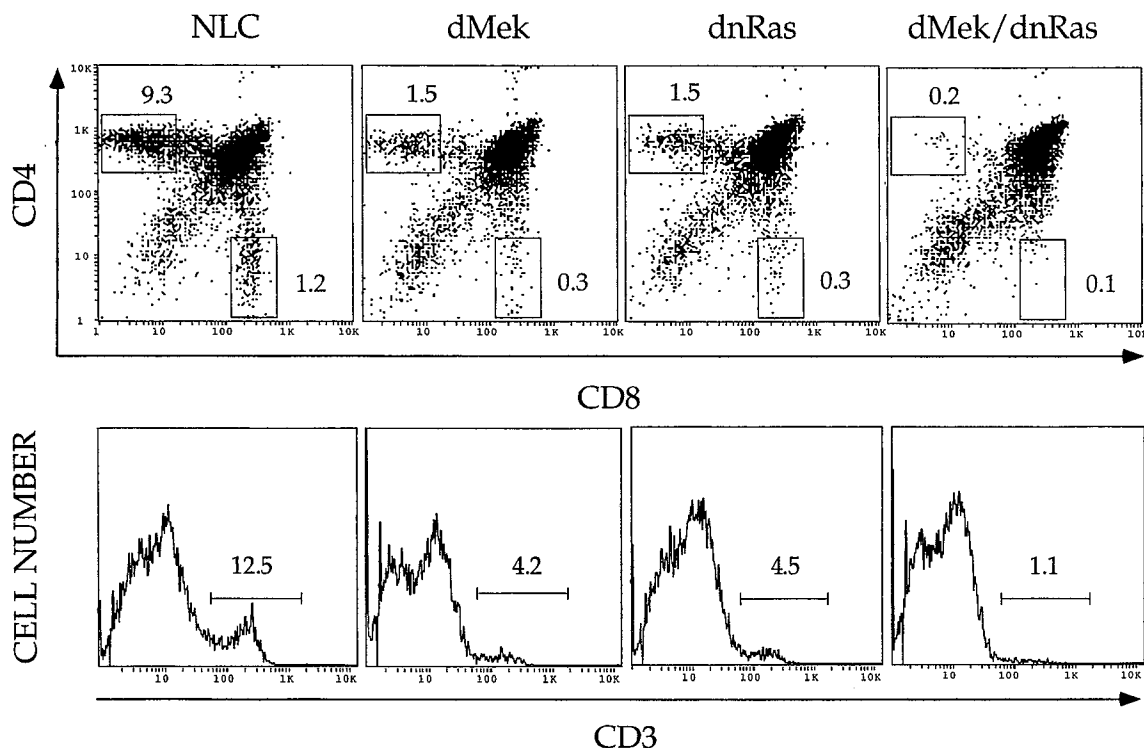

Figure 1. dnRas and dMek act in concert to inhibit the generation of CD3^{hi} single-positive thymocytes. Thymocytes from 6-wk old mice derived from the cross of dnRas (line 15465) × dMek (line A1665) transgenic animals were stained with anti-CD4-PE, anti-CD8-FITC, biotinylated anti-CD3ε, and streptavidin-Tricolor, and analyzed by flow cytometry as described in Materials and Methods. The presence of the transgenes was determined by PCR and Western blotting. Shown are histograms for a normal littermate control (*NLC*), single transgenic animals expressing dnRas or dMek, and a double transgenic animal (*dMek/dnRas*). (*Top*) Two-color, CD4 vs CD8, histogram. The percentage of single-positive thymocytes in the boxed areas is indicated. (*Bottom*) Representation of CD3^{hi} cells in the thymuses of the different animals. The experiment shown is representative of more than five litters examined.

rupted. This was achieved by simultaneously introducing a third transgene, H-Yαβ, encoding a TCR of known specificity that recognizes a male-specific antigen in the context of H-2Dᵇ. Previous studies document the utility of this model system for the analysis of both positive and negative selection events. In adult male animals, virtually all thymocytes are deleted during development through interaction of the H-Y-specific TCR with cognate ligand, whereas in female mice, selection of CD4⁻8⁺ class I–restricted thymocytes proceeds efficiently (11, 17). Triply transgenic mice were obtained by crossing mice heterozygous for dMek (line A1665) to mice bearing a dnRas transgene (line 15728) and the transgene encoding the H-Y TCR (previously bred onto a C57BL/6 background). Progeny of these crosses were genotyped by PCR, and the animals were analyzed by three-color flow cytometry at 4–6 wk of age.

As we have previously shown, the presence of either dn-Ras or dMek dramatically decreases the generation of CD4⁻8⁺ thymocytes in transgenic H-Yαβ female mice.

This effect is best visualized by gating on cells undergoing positive selection that express high levels of the transgene-encoded TCR α chain (18), where a 95% reduction in the proportion of CD4⁻8⁺ cells is typically observed. Coexpression of both dnRas and dMek had very little effect on the already very low levels of mature cells generated in the thymus (Fig. 2). However the increased inhibition of thymocyte development could be appreciated in the spleen, where the number of CD8⁺ T cells was significantly reduced (from 30% of normal in doubly transgenic animals to ≈5–10% of normal in the triply transgenic mice). Despite this dramatic reduction in the generation of CD8⁺ T cells, negative selection in male mice bearing the H-Yαβ transgene remained intact (Fig. 3). These results reinforce the view that the Ras/Raf/Mek/MAP kinase pathway is disproportionately required for effective positive selection.

Quantitative Analysis of Negative Selection Using an OVA-specific TCR. Despite the seemingly normal propagation of negatively selecting signals in the H-Yαβ/dnRas/dMek
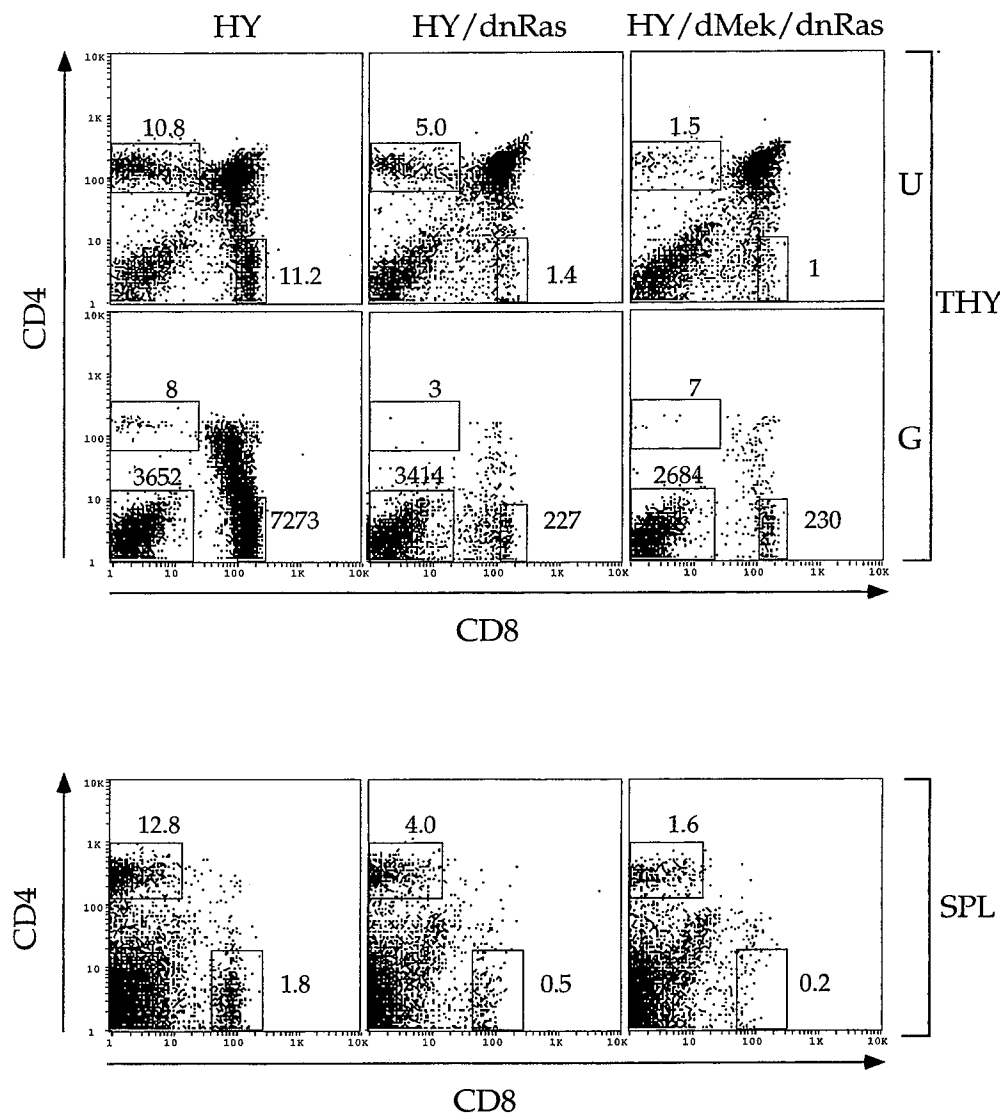
Figure 2. Effect of dnRas and/or dMek on positive selection of thymocytes bearing H-Yαβ TCR. Thymocytes and splenocytes from 6-wk-old littermate female progeny of H-Y/dMek (A1665) × dnRas (15728) or H-Y/dnRas (15728) × dMek (A1665) crosses were stained with anti-CD4-PE, anti-CD8-FITC, biotinylated T3-70 (anti-H-Y TCR α chain) and streptavidin-Tricolor, and analyzed by flow cytometry. Shown are two-parameter histograms of CD4 and CD8 expression in thymocytes (*THY*) and spleenocytes (*SPL*) for the thymocytes, total cells in a live gate (*U*) are compared with those cells present in a T3-70ʰⁱ (H-Y TCR α), small forward scatter gate (G) as described (10). The numbers indicate the percentage of cells in each region in the ungated (*U*) histograms, or the total number of cells from 100,000 (thymus) total events in the gated panel (G). The combination of both dMek and dnRas further inhibits the generation of CD8⁺, H-Y TCR⁺ cells. This is best appreciated in the spleen. Shown is a representative experiment out of three.
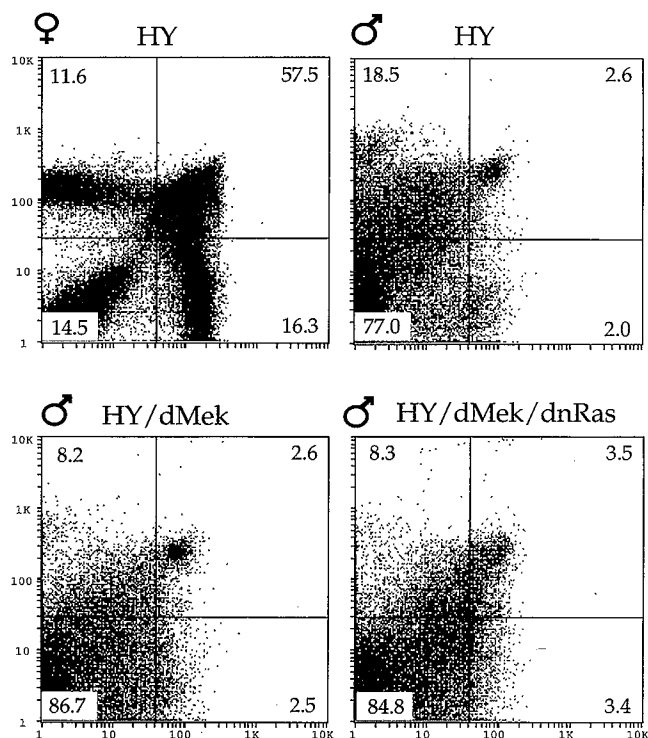
Figure 3. Negative selection of H-Y TCR–bearing thymocytes is not affected by dnRas and dMek. Thymocytes from 6-wk-old littermate male progeny of H-Y/dMek (A1665) × dnRas (15728) or H-Y/dnRas (15728) × dMek (A1665) crosses were stained with anti-CD4-PE, anti-CD8-FITC, biotinylated T3-70 (anti-H-Y TCR α chain), and streptavidin-Tricolor, and analyzed by flow cytometry. The phenotype of the animals was determined by Western blotting and/or PCR as described in Materials and Methods. Shown are two-parameter histograms of CD4 and CD8 expression in thymocytes. The number of thymocytes present in the male animals was similar ($1.2–1.6 \times 10^7$ cells). Shown is a representative litter out of three examined.
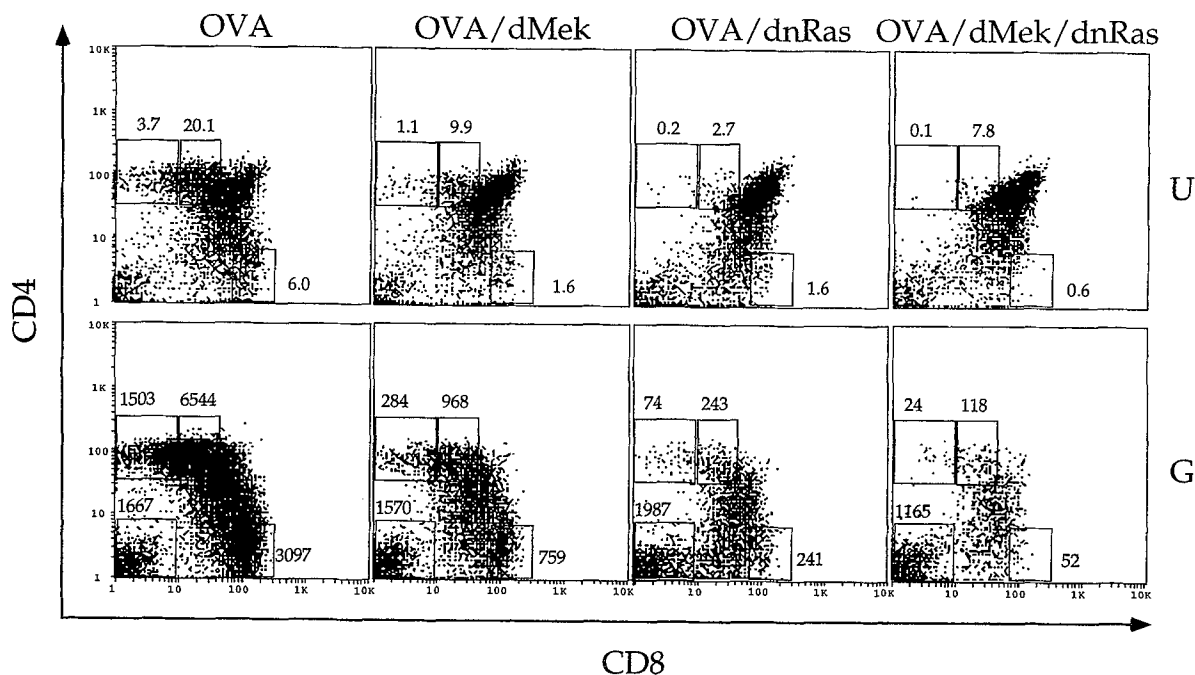
triply transgenic mice, it remained possible that some significant inhibition of these signals was in fact taking place, but that the overall strength of the negative selection signals was too great to permit appreciable reduction in the elimination of cells by Ras pathway blockade. To gain additional insight into the nature of negative selection, we made use of a second TCR transgene (OT-I), derived from an H-2K^b–restricted T cell clone reactive with an octamer peptide (SIINFEKL, termed OVAp) representing residues 257-264 of ovalbumin. This system is especially useful because the negative selection phenomenon can be modeled directly in thymocytes using an in vitro peptide addition system (19). The OVAp peptide will induce T cell activation and in vitro thymocyte deletion at concentrations where only 80 peptide/MHC complexes are formed per presenting cell (20). In addition, variant peptides have been characterized that require somewhat higher (E1) or dramatically higher (V-OVA) concentrations of peptide to induce negative selection (Hogquist, K.A., unpublished results).

Thymic development in OVA (bearing the OT-I transgene), OVA/dnRas, OVA/dMek and OVA/dnRas/dMek animals was analyzed by three-color flow cytometry in

4-wk-old animals. As in other systems, the presence of either the dnRas or the dMek transgene resulted in a 60% decrease in the generation of CD4$^-$8$^+$ thymocytes, and the combination of both transgenes was again much more efficient, decreasing the numbers of mature CD8$^+$ cells by >90%. Gating on cells expressing high levels of the OT-I α chain provided a direct demonstration of the profound inhibition of positive selection, with <2% the normal number of CD8-bearing cells represented (Fig. 4).

An interesting feature of OT-I transgenic animals is the presence in their thymuses of a CD4$^+$CD8^{lo} population which may represent thymocytes that have inappropriately downregulated the correct coreceptor (21). Alternatively, these cells may represent normal intermediates in the commitment pathway for CD4$^-$8$^+$ cells (22, 23). Regardless of their precise provenance, the generation of this population is also dramatically inhibited in the presence of dnRas or dMek, and most profoundly when both transgenes are present, indicating that this early step in the commitment process leading to the generation of single-positive cells requires activation of the MAPK cascade (Fig. 4). It is also notable that, as in the H-Yαβ transgenic animals (8, 10, and Fig. 1), there is a population of transgenic TCR α^{hi}, double-negative cells, products of a distinct differentiative pathway (24) whose generation is not affected by the presence of dnRas, dMek, or both.

Since the combination of dnRas and dMek efficiently blocks positive selection signals generated after ligand occupancy of the OT-I receptor, we tested for impairment of deleting signals derived from the same receptor complex. This was done by incubating total thymocytes for 18 h in the presence of increasing concentrations of test peptides, and thereafter analyzing the representation of viable CD4$^+$8$^+$ cells. Inhibition of negative selection should appear as a shifting of the dose–response curve to the left, such that substantially higher concentrations of test peptide are required to produce an equivalent degree of cell death. Fig. 5 demonstrates that this is not the case. When the maximally stimulatory OVA-p peptide agonist was used, efficient deletion occurred with a K^{del} (half-maximal deletion) value of 10^{-12} M, regardless of whether thymocytes bearing the OT-I transgene alone, or the OT-I transgene with dnRas, dMek, or both, were examined. Hence effective inhibition of the Ras/Raf/Mek/MAP kinase cascade did not affect the fidelity of signaling leading to in vitro deletion when a strong agonist was used. Remarkably, much the same result was obtained when weaker agonist peptides were tested. Thus for E1 (K^{del} = 10^{-8} M), no significant differences in the dose of peptide required for deletion were seen in the doubly or triply transgenic mice (Fig. 5). Hence, if negative and positive selection differ only with respect to the strength of activation of otherwise identical signaling pathways, the magnitude of the difference in strength must be extremely large, sufficient to permit complete blockade of positive selection while leaving intact weak negatively selecting signals, which by titration differ by four orders of magnitude from optimally deleting stimuli. Differences of this magnitude would be surprising if signal strength is gov-
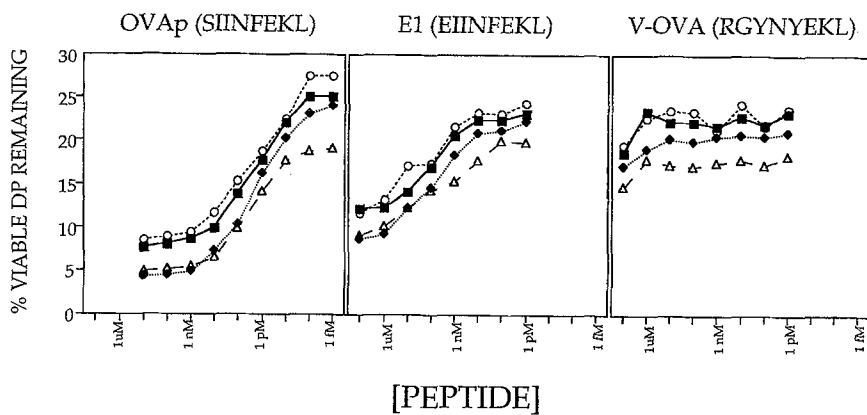
OVA   OVA/dMek   OVA/dnRas   OVA/dMek/dnRas

CD4

10K
1K
3.7   20.1   1.1   9.9   0.2   2.7   0.1   7.8
100
10
                                                                        U
1
6.0   1.6   1.6   0.6

10K
1K
1503   6544   284   968   74   243   24   118
100
10
1667   1570   1987   1165   G
1
3097   759   241   52

1   10   100   1K   10K   10   100   1K   10K   10   100   1K   10K   10   100   1K   10K

CD8

Figure 4. Inhibition of positive selection of thymocytes bearing the OT-I TCR by dnRas, dMek, or both. Thymocytes from 4-wk-old F_1 progeny of homozygous OT-I × dnRas/dMek crosses were stained with anti-CD4-PE, anti-CD8-FITC, biotinylated B20.1 (anti-Vα2 specific, to identify cells bearing the OVA-specific transgenic TCR), and streptavidin-Tricolor, and analyzed by flow cytometry. The phenotype of the animals was determined by Western blotting and/or PCR. Shown are two-parameter histograms of CD4 and CD8 expression from cells in a live gate (U) and cells present in a B20.1^{hi} (OVA-I TCR α), small forward scatter gate (G). The numbers indicate the percentage of cells in each region in the ungated (U) histograms, and total number of cells from 100,000 total events in the gated panels (G). The combination of dMek and dnRas inhibits the generation of CD8$^+$, B20.1$^+$ cells most efficiently. Note also that the generation of B20.1$^+$ CD4$^-$CD8$^-$ cells is not affected. Shown is a representative experiment out of three.

erned by the affinity of the TCR for its ligand, since measured variations among partial agonists typically fall within a narrow range (25). Importantly, the dnRas and dMek transgenes do not themselves promote thymocyte killing since the extraordinarily weak V-OVA agonist ($K^{del} > 10^{-5}$ M) provoked equivalent deletion in thymocytes from all four types of transgenic mice (Fig. 5).

Inhibition of the Ras-MAPK Pathway Does Not Affect the Development of other T Cell Lineages. Although doubly transgenic dnRas/dMek mice have few thymocytes expressing

high levels of the TCR, some such cells can be detected in these animals, and the numbers of these cells exceed the combined numbers of CD4$^-$8$^+$ and CD4$^+$8$^-$ cells (Fig. 1). These observations prompted more detailed evaluation of minority lineages of thymocytes in mice expressing the dominant-negative transgenes.

To analyze the development of $\gamma\delta$ T cells, pregnant females resulting from crosses of dnRas with dMek mice were killed between days E15.5 and E19.5, the fetuses were genotyped by PCR, and the expression of the trans-

OVAp (SIINFEKL)   E1 (EIINFEKL)   V-OVA (RGYNYEKL)

% VIABLE DP REMAINING
30
25
20
15
10
5
0
1uM   1nM   1pM   1fM   1uM   1nM   1pM   1fM   1uM   1nM   1pM   1fM

[PEPTIDE]

Figure 5. Peptide-specific deletion of OVA-TCR–bearing thymocytes proceeds unimpaired in the presence of dnRas, dMek, or both. Thymocytes from 4-wk-old F_1 progeny of homozygous OVA-TCR × dnRas/dMek crosses were incubated overnight with APCs (EL4) that had been preloaded with different peptides, and were then stained with anti-CD4-PE, anti-CD8-FITC, biotinylated anti-CD69, and streptavidin-Tricolor, and analyzed by flow cytometry to determine the number of surviving double-positive cells, as described in Materials and Methods. The phenotype of the animals was determined by Western blotting and/or PCR. Shown are the results obtained after incubating the OVA-TCR$^+$ thymocytes with APCs preloaded with different amounts of the cognate peptide (*OVAp*), a partial agonist peptide (*E1*), and an antagonist peptide (*V-OVA*). An unrelated peptide (poly-Ser) able to bind to K^b did not induce deletion (data not shown). Shown are the results of a representative experiment out of three.

genes was confirmed by immunoblot analysis of thymic lysates. Thymic cellularity was simultaneously assessed by three-color flow cytometry. As shown in Fig. 6, γδ T cells were present in normal numbers in animals expressing dnRas, dMek, or both. Similar results were obtained when the development of Vγ3-expressing thymocytes, a population that ordinarily appears very early in ontogeny (26), was assessed. Appearance of Vγ3-bearing cells requires signals from p56[lck] (27), but was not at all compromised by the presence of the dnRas or dMek transgenes (Fig. 6).

To confirm that these cells were actually expressing the inhibitory transgenes at levels comparable with those seen in αβ thymocytes, we sorted γδ cells from fetal thymuses (E16.5) and measured the expression of Mek protein. As shown in Fig. 7, γδ thymocytes express this protein, 97% of which is transgene encoded, at levels 24-fold higher than

those seen in total thymocytes from normal mice. Since this value is in good accord with the known level of transgene expression in conventional αβ thymocytes, interdiction of the Ras/Raf/Mek/MAP kinase signaling cascade does not appear to compromise development of mature γδ+HSA− cells. Similarly, NK1.1+ αβ T cells, a population of T cells representing an independent lineage that undergoes selection after interaction of the TCR with the CD1 atypical class I molecule (28, 29), develop normally in mice bearing the dnRas and/or dMek transgenes (data not shown). We conclude that activation of the Ras signal transduction pathway is obligatory for maturation of conventional CD4+8− and CD4−8+ thymocytes bearing αβ TCRs, but plays a less important role, and perhaps no role at all, in directing the maturation of other T cell lineages. More remarkably, within the conventional αβ T cell mat-
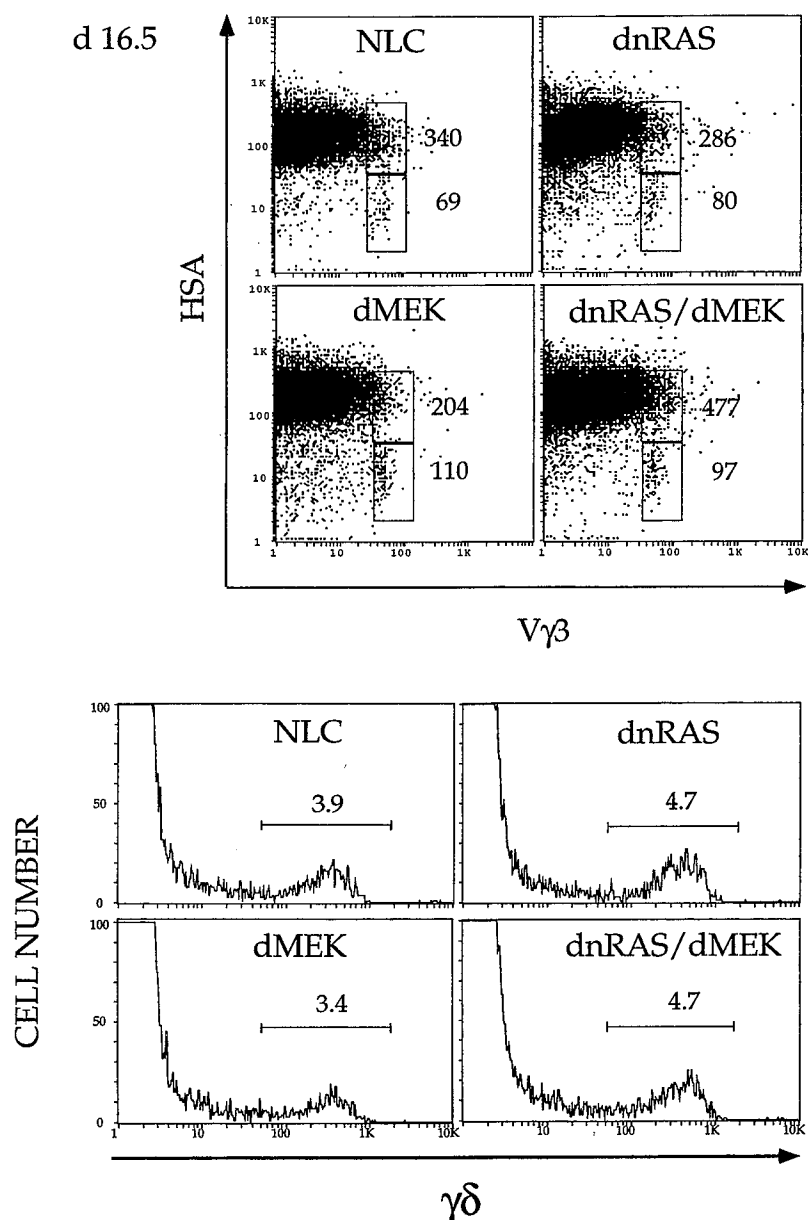
Figure 6. γδ T cells are generated in normal numbers in the presence of dnRas and/or dMek. Timed matings were established by adding one dMek transgenic male to a cage of three dnRas transgenic females for 16 h. The day the males were removed was considered day 0.5. At different time points, pregnant females were killed and the thymocytes from the fetuses stained with a Vγ3-specific antibody and J11d (HSA specific), or with anti-γδ TCR-PE, anti-CD3-FITC, biotinylated anti-TCR β, and streptavidin-Tricolor, and analyzed by flow cytometry. The phenotype of the animals was determined by Western blotting. Shown is a two-parameter stain for Vγ3 and HSA (top) and a single-parameter histogram of total γδ cells (bottom) in 16.5-d fetal thymocytes. The numbers represent (top) total numbers of cells (out of 100,000 events) and the percentage of γδ+ cells (bottom).
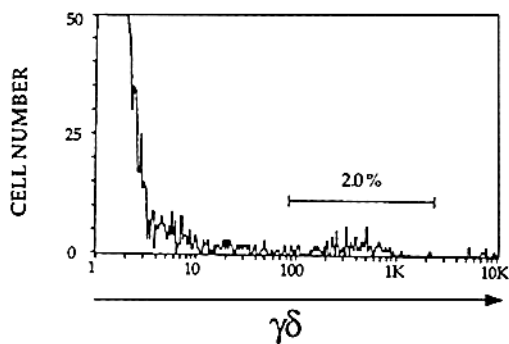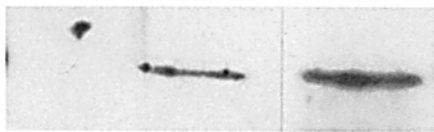
Figure 7. γδ cells from E16.5 normal littermate control and dMek transgenic animals were sorted, washed, counted, and lysed. The lysates, including a separate lysate from normal thymocytes, were analyzed by Western blotting. The density of the bands was quantified by densitometry, yielding the indicated values. Since the A1665 line expresses ~30-fold excess Mek protein (10), the predicted density value from 1.5 × 10⁴ cells would be 2.3.

uration scheme, activation of the MAPK cascade permits positive selection, but need not occur for satisfactory propagation of signals leading to negative selection.

Discussion

The interaction of the TCR with its ligands, molecules of the MHC with bound peptides, may induce different functional consequences in T cells, ranging from proliferation to anergy in mature cells, or from differentiation (positive selection) to apoptosis (negative selection) during development (1). Although recent experiments using altered peptide ligands have shown the importance of the presented peptide in determining the cellular response (3–5, 30), the mechanisms employed by the TCR to impose these different outcomes are not well understood. Recent results indicate that both positive and negative selection are inhibited in thymocytes from animals lacking ZAP-70 (9), suggesting that, at a proximal point from the TCR, a single mutation can block all possible outcomes of TCR engagement. In contrast, our results demonstrate that inhibition of the MAPK cascade defines a branch point in the TCR-derived signal transduction cascade.

Previous genetic and biochemical studies make plain that p21^{ras} sits at a pivotal control point in a serine/threonine kinase signaling pathway. Activation of Ras occurs through

the action of the guanine nucleotide exchange factor Sos, permitting accumulation of Ras in its GTP-bound form. This Ras–GTP complex binds to the Raf kinase and directs it to the plasma membrane where it is activated (31, 32). Raf in turn phosphorylates the MAP kinase Mek-1 (33), which thereafter phosphorylates and activates MAP kinases. These MAP kinases can phosphorylate transcription factors directly, or can impinge upon still other signaling pathways (see reference 15 for a review of this cascade).

Even though expression of either dnRas (8) or dMEK (10) specifically inhibited positive selection, some single-positive cells were still generated in these mice. These cells could represent a population that does not require Ras-derived signals for maturation, or could result from incomplete attenuation of the Ras signaling pathway by the dominant-negative transgene products. Indeed, biochemical analysis showed that the blockade of MAPK activation, although proportional to the amount of dominant-negative transgene expressed, was never complete (10). Here we have shown that simultaneous introduction of both dominant-negative transgenes blocks maturation of single-positive thymocytes almost entirely. Since Ras and Mek participate in the same signaling pathway from the TCR, and since the combined effects of the transgenes mimic what would be expected from each acting independently, we conclude that maturation of conventional CD4$^+$8$^-$ or CD4$^-$8$^+$ αβ T cells from CD4$^+$8$^+$ progenitors absolutely requires signaling via the Ras-initiated kinase cascade.

Although it is possible that dnRas and dMek block so-called accessory signals, known to be required for thymocyte maturation, which are triggered by thymic stromal elements, all evidence suggests that it is the TCR-derived signal itself that undergoes attenuation. TCR stimulation, even in double-positive thymocytes, stimulates the MAP kinase cascade (Alberola-IIa, J., unpublished results), hence an accessory signal that acted in this way would provide no information beyond what was communicated by the TCR itself. More to the point, dnRas and dMek block upregulation of CD69 in vivo (8, 10), which is a TCR-dependent event. Hence activation of Ras, almost certainly as a result of a signal transduction process initiated at the TCR, is required for maturation of conventional, αβ T cells.

Abrogation of ZAP-70 signaling seemingly blocks all thymocyte developmental events that require TCR stimulation. In contrast, complete interdiction of the MAPK cascade does not affect TCR-induced negative selection at all. These results cannot easily be made consonant with models that seek to explain the distinction between positive and negative selection based on quantitative variations in the "strength" of the signal (presumably reflecting the total flux of catalytic events) along a single, unbranched pathway. If such were the case, attenuation of this signaling pathway at any point should yield results like those obtained in ZAP-70$^{-/-}$ mice. Hence, with respect to biological effects, the TCR signaling pathway must at least bifurcate downstream of ZAP-70, and at least one of the resulting branches must serve to stimulate the Ras pathway. The existence of branched TCR signaling pathways makes understandable a

process whereby ligand occupancy of the receptor might provoke different outcomes: depending upon the ligand, activation of different signaling branches might be selectively favored, whether as a result of kinetic (34, 35) or allosteric (36) considerations. However Ras activation, though necessary for positive selection, is almost certainly not sufficient, since negatively selecting stimuli also provoke augmented expression of CD69 (Alberola-Ila, J., and K.A. Hogquist, unpublished results), a Ras-dependent event (37). Thus whereas Ras signals must be present, additional signals must also be absent if positive selection is to result.

Together, our studies and those of others suggest a modified view of the process whereby the avidity of peptide/MHC-TCR interactions regulates thymocyte selection. Significant occupancy of the TCR is in all cases required to permit stimulation of downstream effector pathways, notably the Ras/Raf/Mek/MAPK cascade. However, at high stimulator concentrations owing either to prolonged occupancy of individual receptors (the kinetic model) or to conformational changes imposed by agonist ligands that bind with high apparent affinity (the allosteric model), other pathways become activated that lead to cell death. Interestingly, our studies suggest that these pathways act independently. If individual thymocytes responded to the ratio of signals derived from Ras and from other pathways, peptide-mediated apoptosis should actually have occurred more efficiently in thymocytes from dnRas and dMek animals. Our experiments argue that in the matter of negative selection, the ability of the ligand to activate the Ras/Raf/Mek/MAPK cascade is irrelevant. Viewed from this perspective, negative selection appears to involve a specialized, independent signaling pathway that requires ZAP-70 activation but for which ZAP-70 cannot serve as the sole controlling element.

Some support for this assertion already exists. For example, expression of dominant-negative versions of the nur77 orphan steroid receptor in thymocytes suppressed negative selection, without affecting positive selection (38). Furthermore, a superantigen-stimulated cell death signaling pathway associated with phospholipase A_2 activation has been reported in at least one set of T cell clones, and was shown to be unaffected by stimulatory peptide/MHC ligands (39). A similar pathway may well underlie negative selection.

Finally, there is every reason to believe that the distinction that we observe between Ras pathway signaling and the activation of other biochemical responses in thymocytes will be maintained in mature T lymphocytes. Using alternative promoter constructs that direct transgene expression primarily to the peripheral T cell compartment (40), it should be possible to assign these individual signaling pathways to the biological processes that they control.

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