

Tec Family Kinases Modulate Thresholds for Thymocyte Development and Selection

By Edward M. Schaeffer,^{*§} Christine Broussard,^{*} Jayanta Debnath,^{*} Stacie Anderson,^{*} Daniel W. McVicar,[‡] and Pamela L. Schwartzberg^{*}

From the ^{*}National Human Genome Research Institute and the [‡]National Cancer Institute, Frederick Cancer Research and Development Center, National Institutes of Health, Frederick, Maryland 21702; and the [§]Department of Pathology, University of Chicago, Chicago, Illinois 60637

Abstract

Tec family kinases are implicated in T cell receptor (TCR) signaling, and combined mutation of inducible T cell kinase (Itk) and resting lymphocyte kinase (Rlk)/Txk in mice dramatically impairs mature T cell function. Nonetheless, mutation of these kinases still permits T cell development. While *itk*^{-/-} mice exhibit mild reductions in T cells with decreased CD4/CD8 cell ratios, *rlk*^{-/-}*itk*^{-/-} mice have improved total T cell numbers yet maintain decreased CD4/CD8 ratios. Using TCR transgenics and an in vitro thymocyte deletion model, we demonstrate that mutation of Tec kinases causes graded defects in thymocyte selection, leading to a switch from negative to positive selection in *rlk*^{-/-}*itk*^{-/-} animals. The reduction in both positive and negative selection and decreased CD4/CD8 ratios correlates with decreased biochemical parameters of TCR signaling, specifically defects in capacitive Ca²⁺ influx and activation of the mitogen-activated kinases extracellular signal-regulated kinase 1 and 2. Thus, Tec kinases influence cell fate determination by modulating TCR signaling, leading to altered thresholds for thymocyte selection. These results provide support for a quantitative model for thymic development and provide evidence that defects in negative selection can substantially alter thymic cellularity.

Key words: gene-targeted mice • signal transduction • Itk • Rlk/Txk • T cell receptor

Introduction

Development of a proper immune system requires the selection of lymphocytes expressing a useful repertoire of antigen receptors that can respond to foreign or dangerous antigens but not to self. For T cells developing in the thymus, these selection processes include both positive and negative selection of immature CD4⁺CD8⁺ cells, helping shape the mature T cell repertoire. These processes are thought to be regulated in large part through the interactions between the TCR expressed on a given thymocyte and peptides presented in the context of either class I or II MHC molecules (for review see reference 1).

Several models have been put forth to account for how these distinct selection processes result from signals from the same receptor (for review see reference 1). One model suggests that the strength of the signal generated through the TCR in developing thymocytes affects the development of the T cell repertoire. Strong signals that result from high affinity or avidity interactions between the

TCR and MHC plus peptide lead to negative selection and death, presumably because these types of signals arise from self-antigens present in the thymus. Intermediate or weak signals from the TCR result in survival and maturation (positive selection) of cells that will expand and populate the periphery. Thus, signals that fail to effectively activate peripheral cells can promote development of thymocytes. Absent or minimal signals, such as when cells express a TCR that does not interact with the appropriate MHC, lead to death by neglect.

Alternatively, other models suggest that specific molecules influence selection choices (1). According to this view, qualitative differences in signaling could account for choices between positive and negative selection processes. For example, expression of dominant-negative mitogen-activated protein (MAP)¹ and extracellular signal-regulated kinase (ERK) kinase (MEKK) or Ras mutants alter positive

Address correspondence to Pamela L. Schwartzberg, Bldg. 49/4A38, 49 Convent Dr., National Human Genome Research Institute, NIH, Bethesda, MD 20892-4472. Phone: 301-435-1906; Fax: 301-402-2170; E-mail: pams@nhgri.nih.gov

¹Abbreviations used in this paper: DP, double-positive; ERK, extracellular signal-regulated kinase; GRB, growth factor receptor-bound protein; HSA, human serum albumin; LAT, linker of activated T cells; MAP, mitogen-activated protein; PI, propidium iodide; PLC, phospholipase C; SP, single-positive; WT, wild-type; XLA, X-linked agammaglobulinemia.

but not negative selection, suggesting that activation of specific downstream molecules may contribute to selection decisions in the thymus (2, 3). However, as different molecules downstream of the TCR may be activated by different thresholds of signaling, the two above models may not be mutually exclusive.

The Tec kinases comprise the second largest family of nonreceptor tyrosine kinases with several members expressed specifically in cells of the hematopoietic lineages (4). Mutations of the Tec family kinase Bruton's tyrosine kinase (Btk) are associated with a profound B cell immunodeficiency, X-linked agammaglobulinemia (XLA), with defective B cell development and Ig secretion resulting from impaired signaling from surface IgM (5, 6). In T cells, at least three Tec kinases are expressed: Tec, a widely expressed kinase, and resting lymphocyte kinase (Rlk)/Txk and inducible T cell kinase (Itk), which have expression primarily restricted to the T cell lineage and mast cells. Despite the profound B cell developmental block observed in patients with XLA and a milder block in B cell development in the corresponding mutant *xid* mice, mice with mutations of Rlk/Txk or Itk still develop near normal numbers of T cells (7, 8). In *rlk*^{-/-} mice, T cell numbers and patterns are similar to wild-type (WT) mice (7). Targeted disruption of *itk* leads to only mild reductions in T cell numbers, preferentially affecting the CD4 subset, so that a decreased CD4/CD8 ratio is observed (7, 8).

To address the function and possible redundancy of Tec family kinases in T cells, we generated double knockouts of Rlk/Txk and Itk. These mice still develop mature T cells, but when compared with cells from either *rlk*^{-/-} or *itk*^{-/-} mice, *rlk*^{-/-}*itk*^{-/-} T cells respond very poorly to TCR stimulation (7). Despite more severe defects in TCR-induced proliferation and cytokine production, *rlk*^{-/-}*itk*^{-/-} mice have increased mature T cell numbers compared with Itk-deficient mice. Nonetheless, they maintain a decreased CD4/CD8 cell ratio, suggesting that *rlk*^{-/-}*itk*^{-/-} thymocytes may undergo altered developmental processes.

To examine T cell development in mice deficient in Tec family kinases, we have evaluated thymic development using TCR-transgenic mice and an *in vitro* model of deletion. We demonstrate here that mutation of these Tec family kinases alters both positive and negative selection. The reduction in both positive and negative selection and the decreased CD4/CD8 ratio correlate with decreased biochemical parameters of TCR signaling, including defects in capacitive Ca²⁺ influx and ERK activation. Our results suggest that alterations in TCR signaling resulting from mutation of Tec kinases may change both thymic selection and lineage commitment by altering thresholds for selection and implicate Tec kinases as key modulators of TCR signaling required for normal thymic development.

Materials and Methods

Mice. *rlk*^{-/-}, *itk*^{-/-}, and *rlk*^{-/-}*itk*^{-/-} mice were previously described. *itk*^{-/-} mice were a gift from Dr. D. Littman (New York University, New York, NY). Mice used in this study had

been backcrossed to C57Bl6/J for four generations and were maintained in sterile isolator cages on autoclaved food and water. HY and AND transgenic mice on a C57Bl/6 (H-2^b) background were a gift of Dr. B.J. Fowlkes (National Institute of Allergy and Infectious Diseases, Bethesda, MD). *rlk*^{-/-}*itk*^{-/-} mice were interbred with TCR-transgenic mice, and the F1 progeny were interbred to generate transgene-positive mice in the control, *rlk*^{-/-}, *itk*^{-/-}, and *rlk*^{-/-}*itk*^{-/-} backgrounds.

Antibodies and Reagents. Antibodies for flow cytometry were obtained from PharMingen unless otherwise noted. The following reagents were gifts from the people noted in parentheses: FITC-conjugated T3.70 directed against the HY clonotypic TCR (Dr. E.W. Shores, Food and Drug Administration, Bethesda, MD); anti-CD28 ascites (Drs. J. Powell and R. Schwartz, National Institute of Allergy and Infectious Diseases, Bethesda, MD); anti-CD8 α (83-12-5; Dr. A. Singer, National Cancer Institute, Bethesda, MD); growth factor receptor-bound protein (GRB)-2-glutathione-S-transferase (GST) protein fusion constructs (Dr. R. Wange, National Institute of Aging, Baltimore, MD); anti-Zap-70 and TCR- ζ antibodies (Dr. L. Samelson, National Cancer Institute, Bethesda, MD); and HY peptide (Dr. M. Vacchio, National Cancer Institute, Bethesda, MD). Antibodies directed against Vav, Cbl, and ERK 1 and 2 were obtained from Santa Cruz Biotechnology, Inc., antiactive ERK antibodies were from Promega and Santa Cruz Biotechnology, Inc., antiphosphotyrosine (4G10) and anti-phospholipase C (PLC)- γ 1 (mixed monoclonal) were from Upstate Biotechnology, and anti-phospho-p38 was from New England Biolabs, Inc.

Flow Cytometry. Individual cells were prepared from thymy and stained and analyzed by flow cytometry on a FACScanTM (Becton Dickinson) as previously described (7).

Proliferation Assays. Single-cell suspensions were prepared from spleens of HY-transgenic mice. Red blood cells were lysed in ammonium chloride and then resuspended at 10⁶ per milliliter in the presence of varying numbers of WT irradiated splenocytes incubated with 10 μ M HY peptide (KCSRNRGYL). 48 h later, cells were pulsed with [³H]thymidine and harvested after 8 h.

Cell Death Assays. Individual thymocyte suspensions were plated at 10⁶ in 1 ml of RPMI plus 10% FCS, with 2 mM glutamine, penicillin/streptomycin, and 2-ME on 24-well plates coated with either 10 μ g/ml 2C11 and 10 μ g/ml hamster IgG, 10 μ g/ml anti-CD28, and 10 μ g/ml hamster IgG or both 10 μ g/ml 2C11 and anti-CD28. Alternatively anti-CD28 ascites were used at a 1:500 dilution. Control plates were coated with hamster IgG at 10 μ g/ml. Cells were incubated at 37°C in 5% CO₂ for 18–24 h and then harvested, stained with anti-CD4 and CD8, and analyzed in the presence of 50 μ g/ml propidium iodide (PI). Live cells were collected for a constant time. In other experiments, CD4⁺CD8⁺ double-positive (DP) cells were enriched by panning twice on anti-CD8-coated plates. After panning, cells were 90–95% DP CD4⁺CD8⁺ cells. 2 \times 10⁶ cells were incubated in 500 μ l on a 24-well plate coated with antibodies as above, harvested 16–24 h later, stained with anti-CD4, CD8, TCR- β , and PI at 66 ng per 10⁶ cells, and washed one time before analysis. In these experiments, absolute numbers of live cells recovered were calculated based on the number of live cells (excluding trypan blue) and flow cytometry profiles.

Immunoprecipitation and Western Blotting. Individual cell preparations were resuspended in serum-free media and stimulated on plates coated with 3 μ g/ml 2C11 for varying times. Alternatively, cells were incubated with biotinylated 2C11 and streptavidin (Sigma-Aldrich) and lysed after indicated times. Cells were either lysed in 2 \times protein sample buffer (2% SDS, 0.1 M Tris, pH 6.8,

20% glycerol) for examination of total tyrosine phosphorylation or alternatively in an equal volume of 1% SDS, and then diluted 10-fold with 1% Triton X-100 in PBS with protease inhibitors (Roche Molecular Biochemicals) and sodium orthovanadate for immunoprecipitations. Lysates were clarified by centrifugation and then incubated with the appropriate antibodies for 3–12 h. Protein A–Sepharose or antimouse Sepharose beads were added for 1 h and then collected by centrifugation. Beads were washed three times in 0.5% Triton X-100 in PBS containing protease inhibitors and sodium orthovanadate and then resuspended in protein sample buffer and separated on 10% Tris–glycine gels, transferred to nitrocellulose, and immunoblotted with appropriate antibodies. For affinity precipitations, 25 μ l of glutathione beads bound to GRB-2–GST fusion protein were incubated with cell lysates for 2–4 h and then washed and analyzed as above.

Ca²⁺ Mobilization. Individual cells were isolated and then labeled with Fluo-3 AM and Fura-Red AM (Molecular Probes) for 30 min. Cells were washed and held in the dark and then resuspended in PBS with Ca²⁺ either in the absence or presence of 2 mM EGTA. Baseline Ca²⁺ measurements were recorded, and then cells were stimulated with either biotinylated 2C11 followed by streptavidin or by thapsigargin. Measurements were recorded for at least 10 min and analyzed with Multitime Software (Phoenix Flow Systems).

Results

Altered Thymocyte Populations in *itk*^{-/-} and *rlk*^{-/-}*itk*^{-/-} Mice. To evaluate the roles of Tec family kinases in T cell developmental processes, we examined populations of thymocytes from mice deficient in Rlk, Itk, or both Tec family kinases. Staining with anti-CD4 and -CD8 indicated that *rlk*^{-/-} mice have apparently normal thymocyte populations, whereas *itk*^{-/-} mice have mildly decreased thymic cellularity (Table I; reference 8). In particular, there are decreased numbers of CD4⁺ single-positive (SP) mature cells with increased numbers of CD8⁺ SP mature cells in *itk*^{-/-} mice,

so that an altered CD4/CD8 ratio is observed (Fig. 1). Surprisingly, despite their more severe defects in TCR-induced responses in mature T cells, *rlk*^{-/-}*itk*^{-/-} mice exhibit increased thymic cellularity relative to *itk*^{-/-} mice. However, *rlk*^{-/-}*itk*^{-/-} thymocytes still have decreased CD4/CD8 ratios similar to that observed in *itk*^{-/-} mice. Thus, although they exhibit decreased numbers of CD4⁺ cells, both *itk*^{-/-} and *rlk*^{-/-}*itk*^{-/-} mice have increased total numbers of CD8⁺ SP cells compared with WT animals.

To further examine parameters of thymic development, we examined other markers of T cell development (Fig. 1). Whereas TCR levels on DP thymocytes were similar to those of WT cells, both *itk*^{-/-} and *rlk*^{-/-}*itk*^{-/-} mice exhibited decreased TCR expression on mature cells. Nonetheless, these SP cells appear mature, as indicated by low levels of expression of human serum albumin (HSA; data not shown). More notably, expression of the cell surface marker CD5, which correlates with strength of TCR engagement (9), was decreased severalfold in *itk*^{-/-} and *rlk*^{-/-}*itk*^{-/-} mice. This difference was observed in both SP and CD4⁺CD8⁺ DP cell populations but was most pronounced in the DP cells, a population first undergoing engagement of the TCR. The decreased CD5 expression parallels the observed decrease in responsiveness to TCR stimulation in mature cell populations, arguing that thymocytes as well as peripheral cells have decreased intensity of signaling through the TCR.

Decreased Positive Selection. The altered patterns of mature cell populations combined with the increased numbers of mature T cells in *rlk*^{-/-}*itk*^{-/-} compared with the *itk*^{-/-} animals suggested that processes of T cell development may be distinct in mice deficient in these kinases. To examine processes of thymic selection, we interbred *rlk*^{-/-}*itk*^{-/-} animals with transgenic mice expressing TCRs directed against either the male-specific antigen HY (HY; reference 10) or a peptide from pigeon cytochrome c (AND; reference 11). Expression of these transgenes allows examina-

Table I. Thymocyte Cell Populations

Genotype	<i>n</i>	No. cells	CD4 ⁻ CD8 ⁻	CD4 ⁺ CD8 ⁺	SP CD4 ⁺	SP CD8 ⁺	CD4/CD8
		$\times 10^6$					
WT	9	136 \pm 23	6.8 \pm 3.5 5.0 \pm 2.6%	113 \pm 6.5 83.5 \pm 4.8%	10.8 \pm 2.8 8.0 \pm 2.1%	4.9 \pm 1.5 3.6 \pm 1.1%	2.2
<i>rlk</i> ^{-/-}	6	151 \pm 35	8.7 \pm 4.8 5.8 \pm 3.2%	125 \pm 5.1 83.5 \pm 3.4%	12.2 \pm 1.8 8.1 \pm 1.2%	4.4 \pm 1.9 2.9 \pm 1.3%	2.8
<i>itk</i> ^{-/-}	8	97 \pm 23	5.6 \pm 3.3 5.8 \pm 3.4%	76 \pm 3.8 79.0 \pm 3.9%	6.4 \pm 1.4 6.6 \pm 1.4%	7.9 \pm 1.5 8.2 \pm 1.5%	0.8
<i>rlk</i> ^{-/-} <i>itk</i> ^{-/-}	8	119 \pm 49	7.1 \pm 4.2 6.0 \pm 3.5%	89 \pm 7.0 75.0 \pm 5.9%	10.9 \pm 1.9 9.2 \pm 1.6%	11.5 \pm 2.9 9.7 \pm 2.5%	0.95

Upper line for each genotype indicates absolute cell numbers \pm SD. Lower line indicates percentage of thymocytes in each compartment.

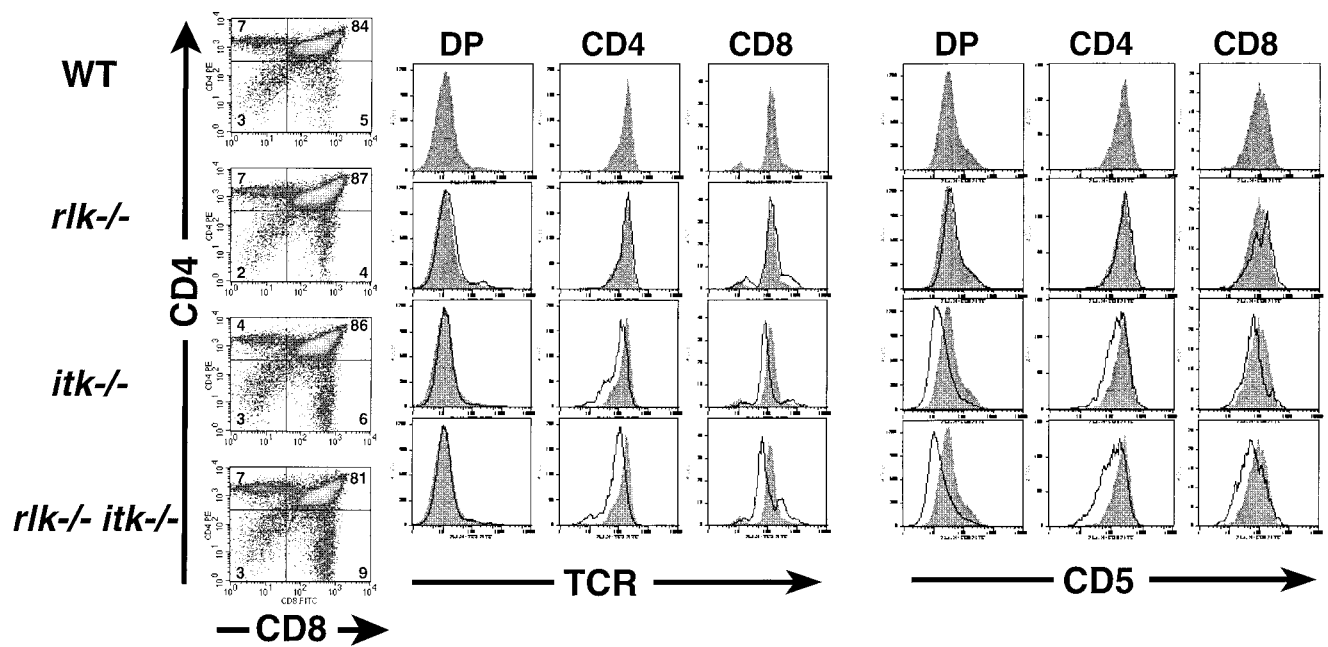


Figure 1. Thymic development of Tec family kinase-deficient mice. Left: thymocytes stained with anti-CD4 and anti-CD8 from WT, *rlk*^{-/-}, *itk*^{-/-}, and *rlk*^{-/-} *itk*^{-/-} mice. Center: histogram of TCR levels from mutant mice (dark lines), overlaid on WT TCR levels (solid gray) for DP and SP CD4⁺ and CD8⁺ cells. Right: histogram of CD5 levels from mutant mice (dark lines), overlaid on WT CD5 levels (solid gray) for DP and SP CD4⁺ and CD8⁺ cells.

tion of positive selection processes in the CD8 and the CD4 lineages, respectively. Furthermore, the HY model allows evaluation of both positive and negative selection.

Thymocytes bearing the AND TCR transgene are positively selected in the I-A^b background, showing large numbers of CD4⁺ cells that express the Vα11 TCR chain (11). In WT AND⁺ mice, virtually all mature SP cells are CD4⁺

and express Vα11. *rlk*^{-/-} AND⁺ mice exhibited varying degrees of subtle defects in this selection process, as indicated by slight reductions in thymic cellularity and absolute numbers of Vα11 CD4⁺ SP cells (Fig. 2 and Table II). Deficiency of *Itk*, however, clearly decreased positive selection, with a marked reduction of Vα11 CD4 SP cells selected and increased numbers of double-negative and

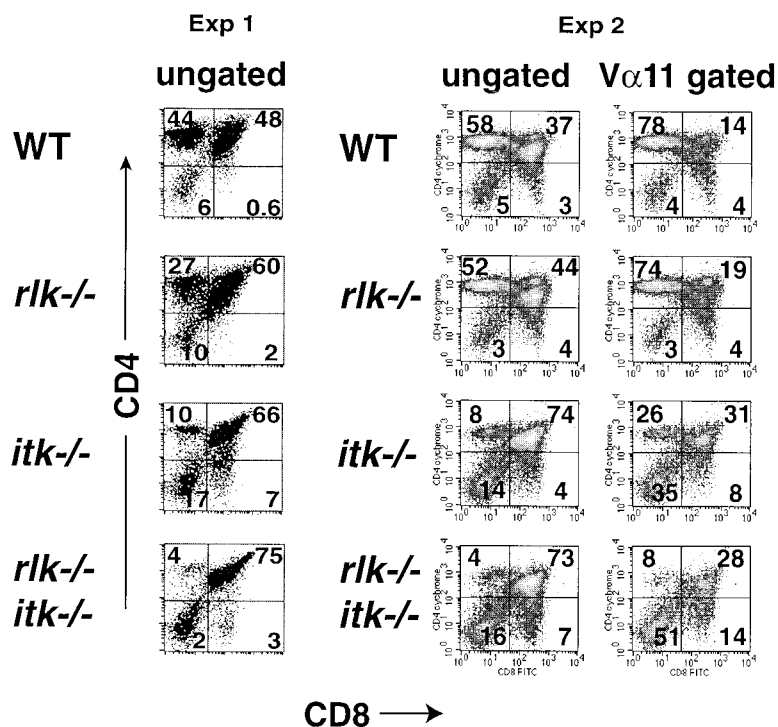


Figure 2. Altered positive selection in *itk*^{-/-} and *rlk*^{-/-} *itk*^{-/-} mice expressing the AND TCR transgene. Thymic profiles stained with anti-CD4 and anti-CD8 from two different experiments (Exp 1 and Exp 2) to show the range of selection in *rlk*^{-/-} animals. Experiment 2 also shows anti-CD4 and CD8 plots gated on Vα11⁺ cells.

Table II. *Altered Selection in AND TCR Transgenics*

	WT	<i>rlk</i> ^{-/-}	<i>itk</i> ^{-/-}	<i>rlk</i> ^{-/-} <i>itk</i> ^{-/-}
Expt. 1	203	163	95.5	113
Expt. 2	300	200	100, 60	65

Total thymocyte numbers $\times 10^6$. Cell numbers are representative of three to five experiments. Expt., experiment.

immature CD8 precursor cells (Fig. 2 and Table II) (8). Similarly, we observed a more severe decrease in positive selection with greater reductions of CD4⁺ cells in mice deficient in both Rlk and Itk. Thus, positive selection in the CD4 lineage with this TCR is most severely impaired in the *rlk*^{-/-}*itk*^{-/-} mice.

As both *itk*^{-/-} and *rlk*^{-/-}*itk*^{-/-} mice have decreased CD4/CD8 ratios compared with WT mice, we wished to also examine a class I-restricted transgene. HY transgenic mice express a class I-restricted TCR directed against the male-specific antigen HY (10). In these mice, selection of the TCR depends on the sex of the mouse. Female HY-transgenic mice positively select the HY TCR into the CD8 cell lineage in the H-2^b background. Deficiency of Rlk again had minimal effects on positive selection, as suggested by the slight decreases in thymic cellularity (Table III). As with the class II-restricted AND transgene, Itk-deficient animals show clear decreases in positive selection of the HY transgene (Fig. 3; reference 8). However, positive selection was even more severely affected by lack of both kinases. Staining of both thymocytes and mature T cells from the HY⁺ females with the T3.70 antibody directed against the clonotypic HY TCR α chain confirmed that positive selection was most severely decreased in the double knockouts, even more significantly than in *itk*^{-/-}*rlk*^{+/-} heterozygous mice (Fig. 3 B).

Table III. *Altered Selection in HY TCR Transgenics*

	HY females			
	WT	<i>rlk</i> ^{-/-}	<i>itk</i> ^{-/-}	<i>rlk</i> ^{-/-} <i>itk</i> ^{-/-}
Expt. 1	122	110	45	38
Expt. 2	191	110	94	73
	HY males			
	WT	<i>rlk</i> ^{-/-}	<i>itk</i> ^{-/-}	<i>rlk</i> ^{-/-} <i>itk</i> ^{-/-}
Expt. 1	10	10	16	30
Expt. 2	7.6	7.6	11	26

Total thymocyte numbers $\times 10^6$. Cell numbers are representative of three to five experiments. Expt., experiment.

*Altered Negative Selection: A Switch to Positive Selection in *rlk*^{-/-}*itk*^{-/-} Mice.* Because the HY antigen is a male-specific antigen encoded by the Y chromosome, male mice expressing the HY transgene can be used to evaluate negative selection. The HY TCR transgene is expressed early in thymic development. Thus, in WT male HY⁺ mice, development of self-reactive cells is arrested so that there are minimal DP cells, and thymic cellularity is reduced ~ 10 -fold (10). In this system, negative selection was not affected by the lack of Rlk. Although it has been previously reported that negative selection is not significantly impaired by deficiency of Itk (8), we observed varying degrees of defects in negative selection in *itk*^{-/-} animals (Fig. 4 A). However, we observed marked effects on negative selection resulting from deficiency of both kinases, with a significant increase in DP and CD8⁺ SP cells in *rlk*^{-/-}*itk*^{-/-} mice (Fig. 4 A).

This defect in negative selection in *rlk*^{-/-}*itk*^{-/-} animals was further confirmed by the cellularity of the thymi from these animals; thymic cellularity of *rlk*^{-/-}*itk*^{-/-} HY⁺ males was increased about threefold relative to WT HY⁺ males (Table III). Not only was negative selection reduced

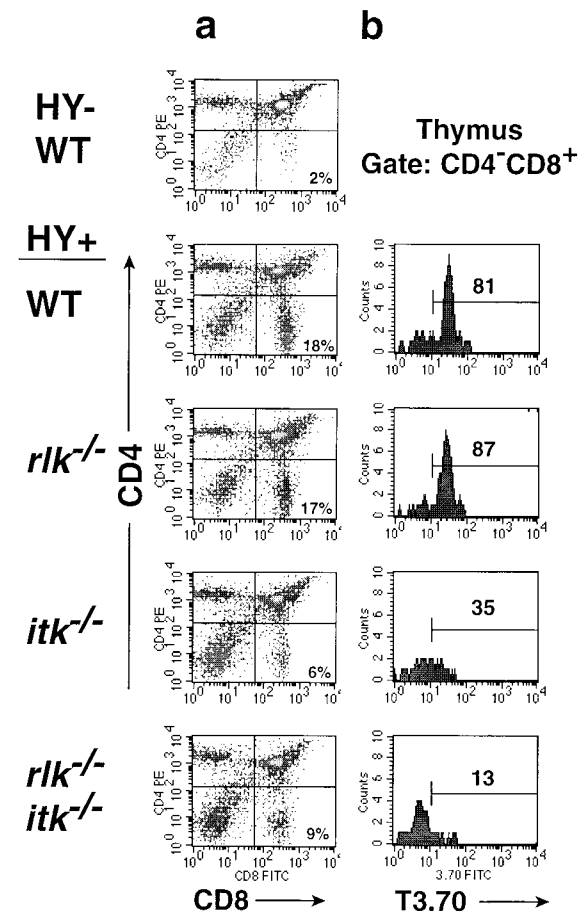


Figure 3. Altered positive selection in female *itk*^{-/-} and *rlk*^{-/-}*itk*^{-/-} mice expressing the HY TCR transgene. (a) Profiles of thymocytes stained with anti-CD4 and anti-CD8. (b) Cells were gated on SP CD8 cells and then examined for T3.70 (anticonotypic TCR antibody) positive cells.

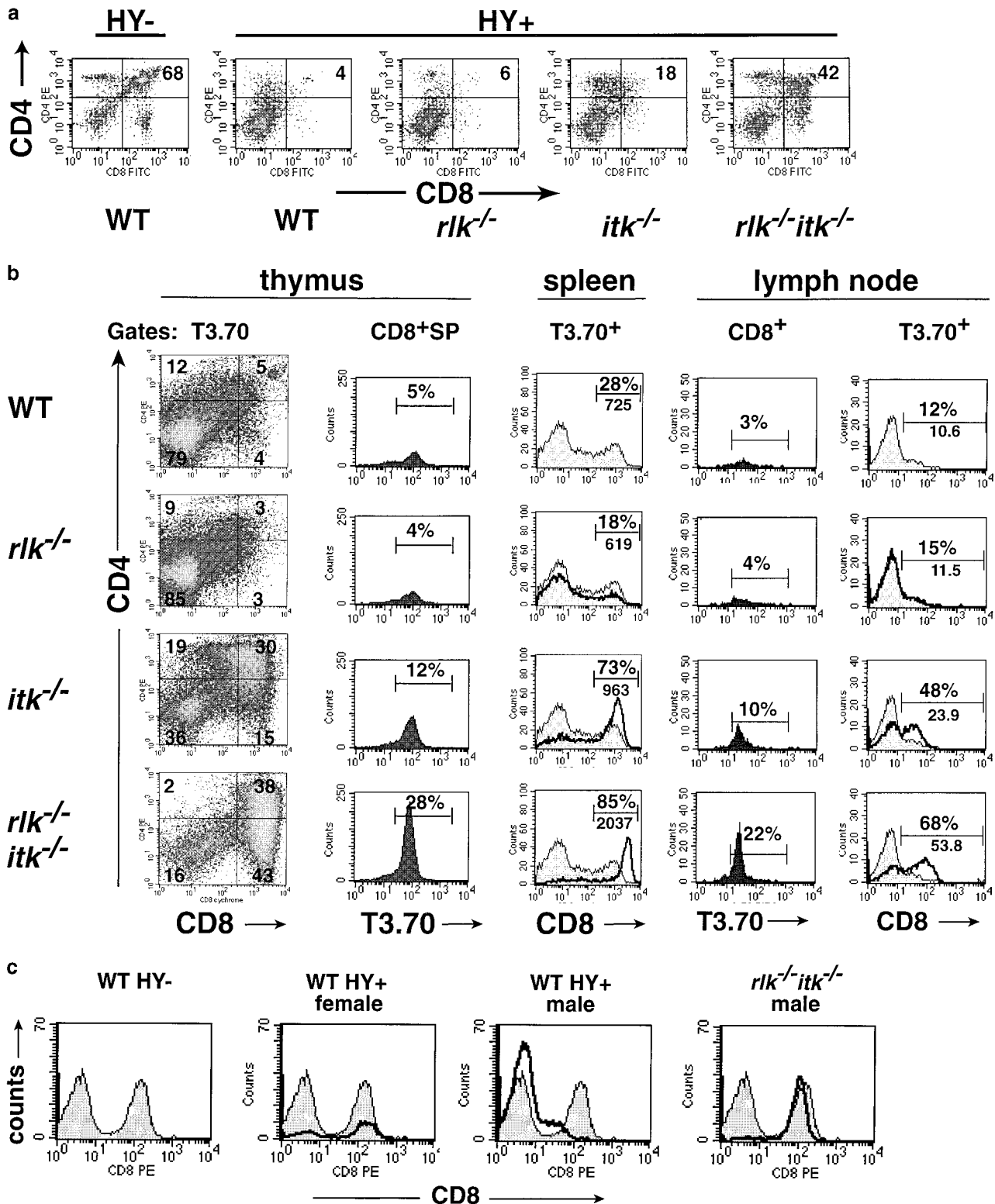
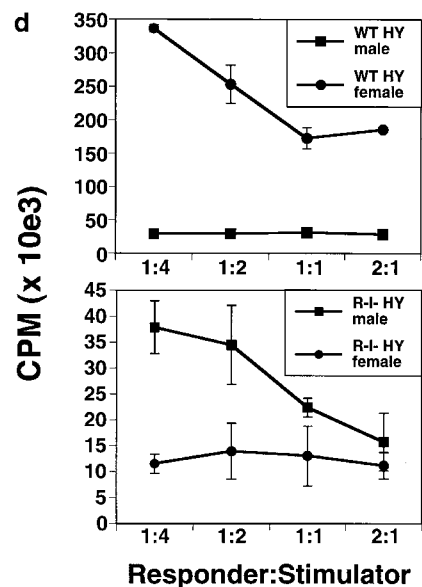


Figure 4. Altered negative selection in male *itk*^{-/-} and *rlk*^{-/-}*itk*^{-/-} mice expressing the HY TCR transgene. (a) Thymic profiles stained with anti-CD4 and anti-CD8. (b) Profiles of cells (separate experiment from a) stained with anti-CD4, anti-CD8, and T3.70 (anticonotypic TCR antibody). First column (from left): thymocytes were gated on T3.70⁺ cells and then examined for CD4 and CD8 expression. Second column: CD8⁺ SP cells examined for T3.70 expression. Numbers indicate percentage of total thymocytes. Third column: splenocytes were gated on T3.70⁺ cells and then examined for CD8 levels. Histograms from mutant mice are overlaid on a WT HY⁺ male (solid gray) for comparison. Numbers above line indicate the percentage of T3.70⁺ cells expressing CD8. Numbers below line indicate mean fluorescence intensity. Fourth and fifth columns: LN cells gated and analyzed as above. LNs are from a separate experiment. (c) CD8 histograms of splenocytes from WT HY⁺ female and male and *rlk*^{-/-}*itk*^{-/-} HY⁺ male. (continues)

in $rlk^{-/-}itk^{-/-}$ HY⁺ male mice, but we observed a relative increase in CD8⁺ SP cells, in particular of those expressing the HY TCR (Fig. 4 B). In WT male HY⁺ mice, the T3.70 cells that mature and populate the periphery express decreased levels of the coreceptor CD8 and are functionally anergic. Unlike WT male HY mice, HY⁺ $rlk^{-/-}itk^{-/-}$ males have increased numbers of T3.70⁺ cells in the periphery that express near normal levels of CD8 (Fig. 4, B and C). The profile of these peripheral cells is therefore remarkably similar to that observed in WT HY females that have undergone positive selection (Fig. 4 C). Together, these results are suggestive of a switch from negative selection to positive selection in the double-knockout mice. Furthermore, these peripheral cells in HY⁺ $rlk^{-/-}itk^{-/-}$ males can respond to the HY antigen, as demonstrated by proliferation assays using the HY peptide and APCs as stimuli (Fig. 4 D), although they respond very poorly, consistent with the abnormal responses in TCR-mediated signaling seen in $rlk^{-/-}itk^{-/-}$ mature T cells. These results suggest that the increased thymic cellularity in $rlk^{-/-}itk^{-/-}$ mice, compared with $itk^{-/-}$ mice, could result from defects in negative selection and that reduction in TCR signaling can lead to a paradoxical increase in thymic cellularity.

Decreased In Vitro Deletion of Thymocytes. To further examine negative selection, we used an in vitro model of thymocyte deletion induced by plate-bound anti-CD3 and



(continued) Histograms from mutant mice are overlaid on a WT non-HY female (solid gray) for comparison of CD8 levels. (d) Proliferation of splenocytes from HY⁺ transgenic mice. Top: proliferation of WT HY⁺ splenocytes incubated with varying ratios of irradiated WT APCs plus HY peptide. Bottom: proliferation of $rlk^{-/-}itk^{-/-}$ HY⁺ splenocytes incubated with varying ratios of irradiated WT APCs plus HY peptide. Different scales are used in the top and bottom panels due to the poor proliferative responses of $rlk^{-/-}itk^{-/-}$ T lymphocytes. Note that WT female HY⁺ splenocytes proliferate to the HY antigen in a dose-responsive fashion. In contrast, $rlk^{-/-}itk^{-/-}$ HY⁺ male splenocytes proliferate to the HY antigen (whereas the nonselected female cells fail to proliferate).

-CD28 antibodies, a strong stimulus that has been shown to drive apoptosis in the majority of DP thymocytes in culture (12). As shown in Fig. 5 A, cell death induced by anti-CD3 plus anti-CD28 was decreased in $rlk^{-/-}itk^{-/-}$ cells, consistent with our in vivo observations using the HY model.

Surprisingly, treatment of either the $itk^{-/-}$ and $rlk^{-/-}itk^{-/-}$ thymocytes with anti-CD3 and anti-CD28 gave rise to altered cell populations. Engagement of the TCR in WT or Rlk-deficient cells caused a decrease of DP cells concurrent with engagement of the TCR. However, in both the $itk^{-/-}$ and double-knockout genotypes, we observed a shift in cell populations so that there was a marked increase in the numbers of CD4⁺ cells present in $rlk^{-/-}itk^{-/-}$ mice (Fig. 5 B).

To determine whether this CD4⁺ population resulted from proliferation of mature CD4⁺ SP cells, we enriched for CD4⁺CD8⁺ DP cell by panning with anti-CD8 and performed similar experiments. In this cell population, we again saw decreased cell death in the $rlk^{-/-}itk^{-/-}$ cells, as apparent both by the percentage PI⁺ cells and the absolute number of viable DP cells (Fig. 5 C). Intermediate defects were observed in cells from $rlk^{-/-}$ and $itk^{-/-}$ mice. In contrast, neither dexamethasone- (data not shown) nor anti-Fas-mediated cell death were reduced in thymocytes from the Tec-deficient mice, arguing that the decreased cell death results specifically from defects in TCR/CD28-mediated responses. Furthermore, we also observed a striking increase in absolute numbers of CD4⁺ cells present after 24-h stimulation with anti-CD3 and anti-CD28 (Fig. 5, C and D). For $itk^{-/-}$ cells, CD4 levels remained relatively low, whereas in the $rlk^{-/-}itk^{-/-}$ cells, CD4 levels were increased. This population of CD4 cells exhibited higher levels of α/β TCR than DP cells but lower than CD4 SP cells from ex vivo thymi (Fig. 5 E). Similarly, these CD4 cells had not fully downregulated the immature cell marker HSA but were lower in HSA than DP cells (data not shown). The intermediate levels of TCR, CD4, and HSA are suggestive of a CD4⁺CD8^{lo} stage previously described as a precursor population to both CD4⁺ and CD8⁺ SP cells (13) and is reminiscent of that seen in an in vitro assay for thymocyte differentiation (14). The phenotype of these CD4⁺ cells is therefore suggestive of progression of DP to an intermediate pre-SP stage rather than proliferation of an already mature SP CD4⁺ population. The novel appearance of this CD4 cell population was particularly pronounced in the double knockouts, again suggesting a switch from deletion to a developmental pathway. Thus, a signal that would normally induce death appears to induce survival and differentiation of $rlk^{-/-}itk^{-/-}$ thymocytes.

Biochemical Defects in $rlk^{-/-}itk^{-/-}$ Mice. To evaluate the nature of the defects in the $rlk^{-/-}itk^{-/-}$ thymocytes, we examined signaling events downstream of TCR engagement. Although modest delays in tyrosine phosphorylation of proteins can be observed after anti-CD3 stimulation, total phosphotyrosine patterns were grossly normal (Fig. 6 A). We have previously reported that tyrosine phosphorylation of TCR ζ chain and ZAP-70 was normal in CD3-stimulated mature cells, indicating normal early tyrosine

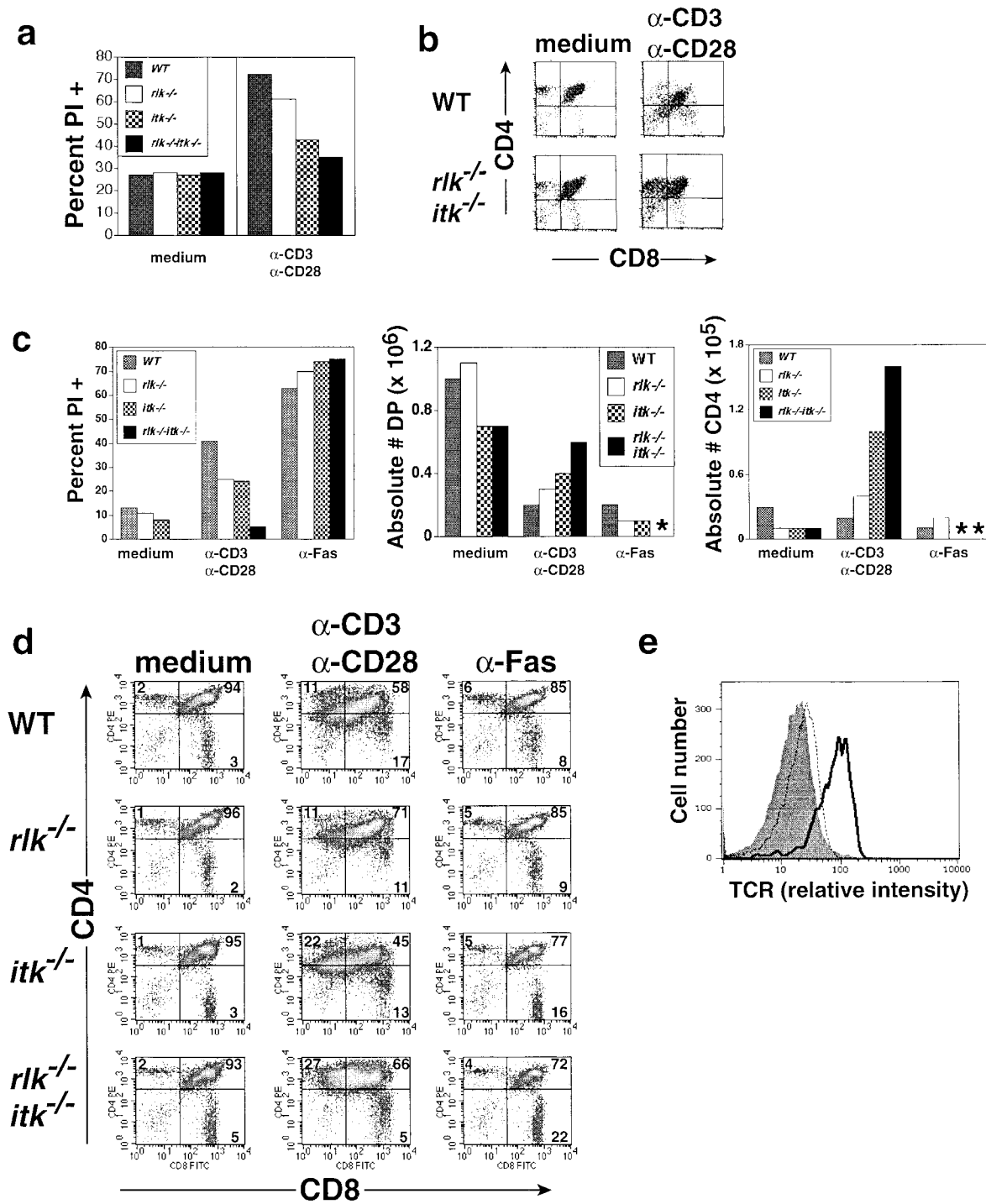


Figure 5. Abnormal responses to death-inducing signals in *itk*^{-/-} and *rlk*^{-/-}*itk*^{-/-} thymocytes. (a) Altered responses to plate-bound anti-CD3 and CD28. Isolated cells from total thymi were plated on anti-CD3 and CD28 for 16–24 h, stained for CD4 and CD8, and analyzed in the presence of PI. (b) CD4 versus CD8 cell analyses of total thymocytes treated as in a. (c) Cells viability (percentage PI⁺), total live DP cells, and total live CD4⁺ cells from enriched DP cell populations treated with plate-bound anti-CD3 and CD28 or anti-Fas for 24 h (* indicates live cell numbers <0.1 on these graphs). (d) Flow cytometry analyses of cells stained for CD4 and CD8 derived from enriched DP cells treated for 24 h. (e) Intermediate low TCR levels on CD4⁺ SP cells from *rlk*^{-/-}*itk*^{-/-} thymocytes after treatment with plate-bound anti-CD3 and CD28. TCR levels of CD4⁺ cells (hatched line) overlaid on TCR levels of DP cells (solid gray) and CD4 SP cells from ex vivo thymi (dark line).

phosphorylation events (7). Similarly, tyrosine phosphorylation of ζ chain and Zap-70 were grossly intact in thymocytes activated with anti-CD3 ϵ (data not shown).

In contrast to normal activation of immediate early TCR signaling events, we observed clear alterations in Ca^{2+} mobilization in response to anti-CD3 stimulation, with graded defects in the *itk*^{-/-} and *rlk*^{-/-}*itk*^{-/-} cells (Fig. 6 B). In peripheral T cells from *itk*^{-/-} and *rlk*^{-/-}*itk*^{-/-} mice, we have observed defects in both the initial peak and prolonged phase of Ca^{2+} mobilization (7, 15). However, in thymocytes, alterations in Ca^{2+} mobilization were limited to the late phase of Ca^{2+} influx. Inclusion of EGTA in the media resulted in nearly identical Ca^{2+} profiles from all genotypes, confirming that the defects were restricted to capacitive Ca^{2+} influx from external sources. Incubation with thapsigargin, an inhibitor of the endoplasmic reticular Ca^{2+} ATPase (16), led to normal Ca^{2+} influx, demonstrating that store-operated Ca^{2+} mobilization was similar in all mice (data not shown) and suggesting that the defect lay in the regulation of Ca^{2+} mobilization. In lymphocytes, Ca^{2+} mobilization is controlled by the activity of PLC- γ (17, 18), which cleaves phosphoinositide 4,5-bisphosphate to diacylglycerol, which activates protein kinase C family members, and inositol triphosphate, which binds to receptors on intracellular organelles, inducing the release of intracellular Ca^{2+} stores and the subsequent activation of capacitive Ca^{2+} influx from extracellular sources (19). Consistent with the defective Ca^{2+} responses in *rlk*^{-/-}*itk*^{-/-} thymocytes, we also observed mild defects in tyrosine phosphorylation of PLC- γ 1 after stimulation with antibodies to CD3 ϵ (Fig. 6 C).

Recently, Tec family kinases have been implicated as part of complexes nucleated by the adaptor molecules linker of activated T cells (LAT) and Src homology 2 domain-containing lymphocyte protein of 76 kD (SLP-76) that are required for activation of PLC- γ and Ca^{2+} mobilization (Fig. 7 A; references 20–24). To examine how deficiency of Tec kinases affects other components of this signaling complex, we looked at the tyrosine phosphorylation of LAT and other GRB-2-associated molecules including SLP-76 and Cbl (25, 26). Again, we observed normal patterns of tyrosine phosphorylation with only modest delays in tyrosine phosphorylation of GRB-2-associated proteins (Fig. 7, B and C). Similarly, tyrosine phosphorylation of Vav, another molecule recruited into this complex (27), was also intact. These data suggest that although Tec kinases contribute to these complexes, phosphorylation and assembly of components of these complexes can occur independently of Tec family kinases.

The LAT-GRB-2/Gads-SLP-76-PLC- γ and Tec family kinase complexes are thought to be critical determinants of both Ca^{2+} mobilization and activation of the MAPK kinases (MAPKs) ERK 1 and 2 (7, 21, 23, 24). Because ERK signaling has been shown to influence CD4 and CD8 lineage commitment (28, 29) and both *itk*^{-/-} and *rlk*^{-/-}*itk*^{-/-} mice exhibit altered CD4/CD8 ratios, we examined whether defects in ERK activation existed in thymocytes from these mice. We observed a reproducible decrease in

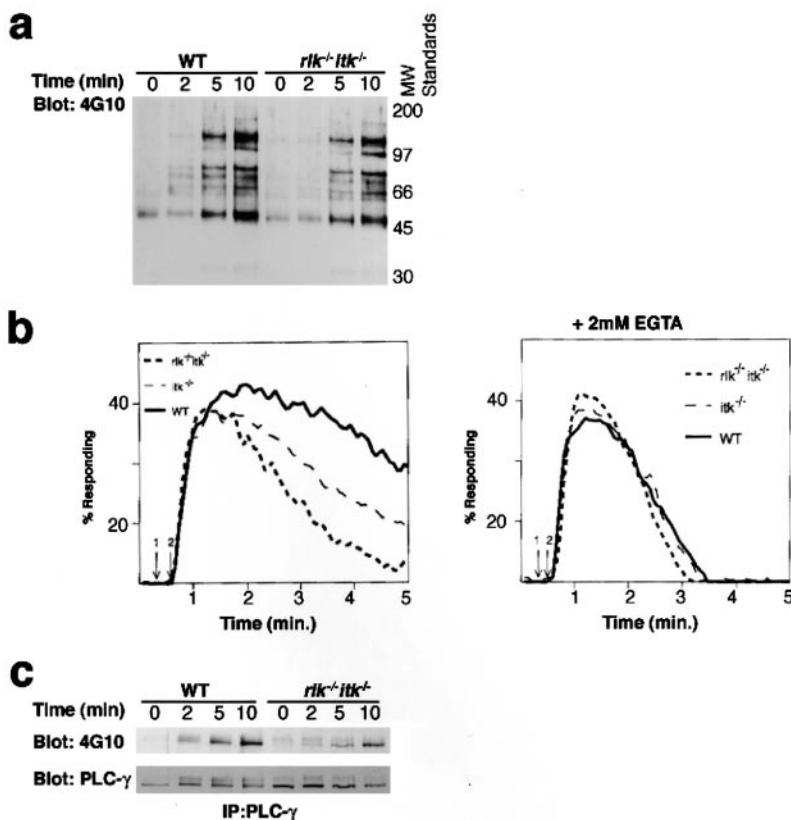


Figure 6. Altered Ca^{2+} mobilization in response to anti-CD3 stimulation in *itk*^{-/-} and *rlk*^{-/-}*itk*^{-/-} cells. (a) Normal patterns of tyrosine phosphorylation after anti-CD3 stimulation of *rlk*^{-/-}*itk*^{-/-} thymocytes. Cells were stimulated with plate-bound anti-CD3 for indicated times and then lysed and analyzed for phosphotyrosine by immunoblotting. (b) *itk*^{-/-} and *rlk*^{-/-}*itk*^{-/-} thymocytes exhibit decreased Ca^{2+} mobilization in response to TCR stimulation. First arrow, addition of biotinylated anti-CD3; second arrow, addition of streptavidin. Cells were analyzed in the presence (left) or absence (right) of EGTA. (c) Altered PLC- γ phosphorylation in *rlk*^{-/-}*itk*^{-/-} thymocytes in response to TCR stimulation. Lysates from cells stimulated as above were immunoprecipitated with anti-PLC- γ and immunoblotted with antiphosphotyrosine.

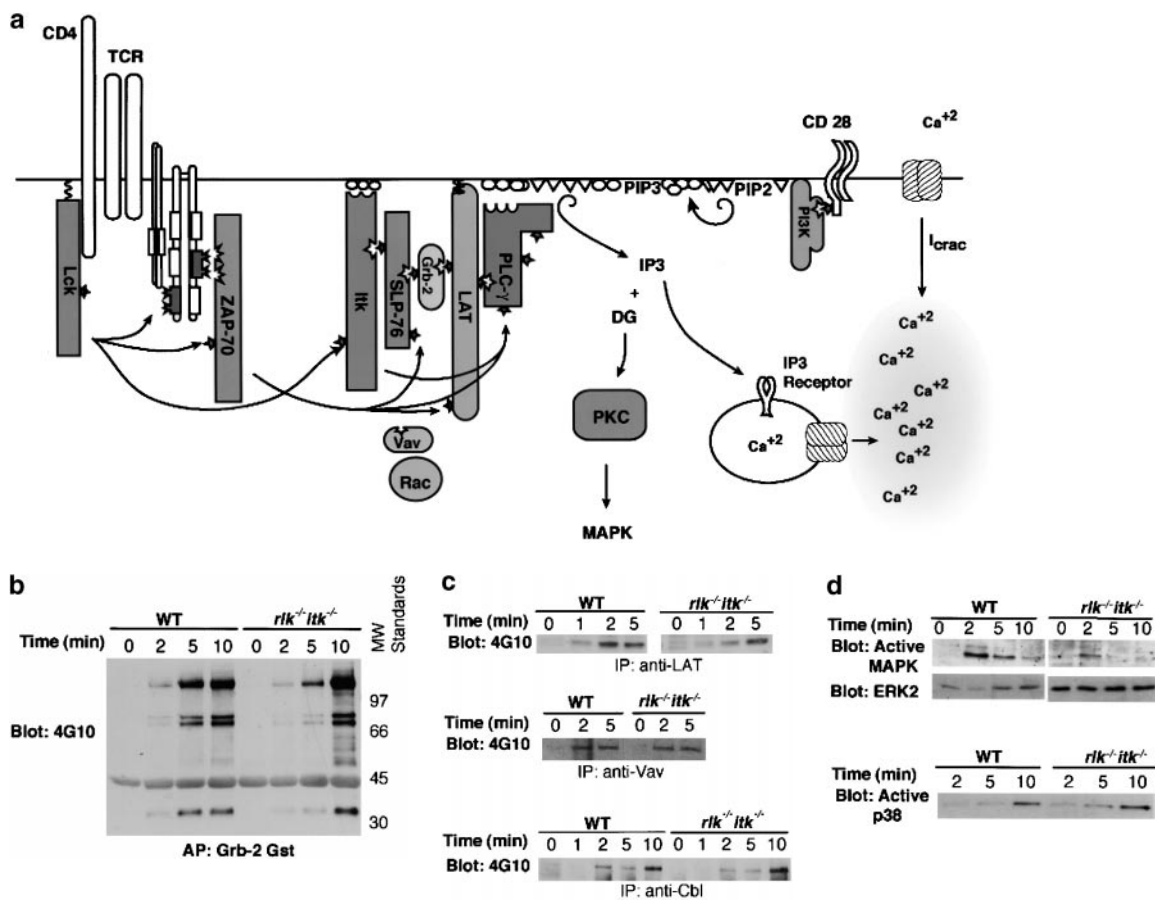


Figure 7. Analyses of proteins in Ca^{2+} -MAPK complex: normal tyrosine phosphorylation of components of Ca^{2+} complex, but decreased activation of ERKs. (a) Complex of LAT-associated proteins required for normal Ca^{2+} mobilization and activation of ERKs. (b) Normal patterns of tyrosine-phosphorylated proteins associated with GRB-2, including LAT (36–38 kD), SLP-76 (76 kD), and Cbl (120 kD) after anti-CD3 stimulation. Cells were stimulated for indicated times on plate-bound anti-CD3 and lysed, and clarified lysates were incubated with GRB-2-GST on glutathione beads. Affinity-purified proteins were immunoblotted with antiphosphotyrosine. (c) Normal tyrosine phosphorylation of LAT, Cbl, and Vav after anti-CD3 stimulation. Cells were stimulated for indicated times, and immunoprecipitated proteins were immunoblotted with antiphosphotyrosine. (d) Altered activation of the MAPKs ERK 1 and 2. Cells were stimulated with anti-CD3, and lysates were analyzed for dually phosphorylated active ERK 1 and 2 and total levels of ERK 2. Bottom panel, lysates were analyzed with antiactive (phospho)-p38.

ERK activation in response to anti-CD3 stimulation in both *itk*^{-/-} and *rlk*^{-/-itk}^{-/-} thymocytes (Fig. 7 D), consistent with the decreased number of CD4 SP cells present in these mice. In contrast, activation of p38, a MAPK shown to influence negative selection (3), appears relatively normal in thymocytes from these mice (Fig. 7 D), suggesting that activation of this MAPK is independent of PLC- γ activation.

Discussion

Using mice with targeted mutations of Rlk, Itk, or both T cell-specific Tec kinases, we demonstrate here that modification of T cell signaling through mutation of these kinases leads to altered thymic development. Specifically, we provide evidence that mutation of the Tec kinases in T cells decreases both Ca^{2+} mobilization and MAPK activation in thymocytes and leads to switches from positive selection to death by neglect and from negative selection to positive selection. These results are consistent with a model

in which developmental decisions in the thymus are influenced by diminished signaling through the TCR. Thus, thymocytes that would normally see a weak, positively selecting stimulus register this as a weaker signal in *itk*^{-/-} and *rlk*^{-/-itk}^{-/-} mice and convert positive selection to death by neglect. Likewise, *rlk*^{-/-itk}^{-/-} cells interpret a strong stimulus normally leading to cell death as a weaker signal leading to positive selection. These results correlate with expression levels of CD5 in DP cells from the mutant mice, expression of which has been found to parallel the strength of TCR engagement (9).

It is not clear whether distinct signals mediate positive and negative selection in the thymus. For example, alteration of the MAPK pathway by expression of dominant-negative mutants of Ras and MEKK can alter positive selection with minimal effects on negative selection, whereas interference with p38 or Jun NH₂-terminal kinase activation inhibits negative selection (2, 3). One interpretation of our data is that Rlk contributes a specific signal required for negative selection, either downstream of the TCR or from

coreceptors such as CD28. Although we have not seen evidence of this in the *rlk*^{-/-} mice, either in HY male mice or in an in vitro model of deletion using anti-CD3 and CD28 antibodies, it is also possible that this phenotype may only be revealed in the context of Itk deficiency.

Alternatively, regulation of positive and negative selection may not require distinct signals but rather the same signals operating at different intensities (1). Negative selection may therefore involve the same biochemical intermediates as positive selection but require a stronger or more prolonged signal through the TCR and may be less sensitive to small perturbations in signaling. By this model, slight defects in TCR signaling could minimally affect positive selection while not impairing negative selection. Moderate defects in TCR signaling, as in the *itk*^{-/-} mice, could alter positive selection in the AND and HY models but have a less severe effect on negative selection. The more profoundly impaired TCR signaling in *rlk*^{-/-}*itk*^{-/-} double knockouts, as demonstrated by their defect in Ca²⁺ mobilization (Fig. 6), may not only more severely affect positive selection but also impact negative selection. Similar phenotypes are observed in mice with mutations of the ζ chain of the TCR (30, 31). The graded nature of the defects we observe in positive and negative selection suggests that these processes may be part of a continuum, consistent with a quantitative view of thymic selection processes. It should be noted, however, that these quantitative differences may be translated into differential activation of downstream molecules, perhaps accounting for data supporting both models of selection.

Although we cannot rule out other effects resulting from elimination of both Rlk and Itk, particularly effects on other signaling pathways, our data suggests that diminished TCR signaling can in fact lead to a paradoxical increase in thymic cellularity in *rlk*^{-/-}*itk*^{-/-} mice compared with *itk*^{-/-} mice due to decreased negative selection. This increased cellularity has additional implications for the relative numbers of cells undergoing negative selection in the thymus. Estimates of cells undergoing negative selection have varied widely depending on the system used to examine this question (32–36). Our results suggest that defects in this process can significantly influence cell number and lead to increased thymic cellularity.

The development of cells that would normally be deleted, as in the *rlk*^{-/-}*itk*^{-/-} HY⁺ male mice, also suggests that reduction of signal may lead to the development of autoreactive cells, a process that may contribute to the development of autoimmunity in immunocompromised hosts. Nonetheless, the potentially self-reactive cells that develop in young male *rlk*^{-/-}*itk*^{-/-}HY⁺ mice respond poorly to self HY antigen and do not show evidence of activation markers, including CD69 and CD25 (data not shown), consistent with the cellular and biochemical defects we observe in mature cells. Indeed, in order for these cells to proliferate in vitro, addition of exogenous peptide was required. This decreased reactivity may account for a lack of obvious signs of autoimmunity in these animals. Whether the activity of these escaped self-reactive cells may be altered under certain

conditions, leading to an autoimmune response, remains an interesting question for future exploration. Notably, a high percentage of T cells from *itk*^{-/-} and, even more so, *rlk*^{-/-}*itk*^{-/-} mice express memory cell markers (Schaeffer, E.M., C. Broussard, and P.L. Schwartzberg, unpublished observations). It is interesting to speculate that these memory cells might result from tonic stimulation of TCRs that recognize self-antigens.

Biochemically, the defects we observe are consistent with an emerging picture of Tec family kinases as a component of a signaling complex including LAT and SLP-76 that is required for full PLC- γ activation, Ca²⁺ mobilization, and ERK activation (7, 15, 20, 22). Both SLP-76 and LAT have recently been shown to bind to Itk in an inducible fashion, thereby providing a mechanism for recruiting Tec family kinases into this complex (20, 22). In peripheral cells, the Tec kinases have been implicated in the phosphorylation and activation of PLC- γ . Our biochemical data suggests that, in thymocytes, Tec family kinases are also required for full Ca²⁺ mobilization and MAPK activation downstream of PLC- γ (7, 15, 37–39). However, the relatively normal tyrosine phosphorylation of LAT and the other GRB-2-associated molecules including SLP-76 and Cbl suggest that, unlike the more proximal components, the Tec kinases are probably not required to initiate formation of this complex. It is notable that we have observed mild kinetic delays in tyrosine phosphorylation of components of this complex. Thus, we cannot rule out the possibility that Tec kinases either may contribute to these phosphorylation events or alternatively may be important for the integrity of this Src homology 2-mediated complex. The recent observation that Rlk can phosphorylate SLP-76 supports this notion (40).

Mutation of these kinases, therefore, gives rise to biochemical defects similar to but less severe than that seen in LAT- or SLP-76-deficient cells (21, 23). Thus, mutation of the Tec kinases, unlike that of the more proximal tyrosine kinases or the more proximal components of the Ca²⁺ complex, does not eliminate downstream signaling nor prevent T cell development but merely changes the character or duration of the signal, leading to altered thresholds for selection. This altered selection in response to signaling defects is reminiscent of that observed with altered peptide ligands (APLs) with reduced TCR activation (41–43). Thus, just as activation of a TCR by APLs permits development of thymocytes (44), mutation of the Tec kinases can permit development of cells that might normally be deleted. Proper activation of the Tec kinases may therefore be critical for helping determine selection processes in the thymus.

The defects we observe in Ca²⁺ mobilization are consistent with a role for Tec kinases in regulating capacitive Ca²⁺ entry (45). Inclusion of EGTA generates Ca²⁺ mobilization profiles that are not significantly different than those of WT cells, suggesting that other molecules contribute to the initial activation of PLC- γ , particularly in thymocytes. These results are similar to those observed in B lymphocytes from XLA patients where altered PLC- γ activation lead to defects that are most pronounced in the late

phases of capacitive Ca^{2+} entry (37). Although mutation of *rlk* itself does not lead to significant phenotypes, mutation of this gene substantially worsens the *itk*^{-/-} phenotype (7), and overexpression of Rlk/Txk can partially compensate for the lack of Itk (46, 47), suggesting potential redundancy between these two Tec kinases in T cells. Thus, as in Btk-deficient B lymphocytes, the Tec kinases Itk and Rlk together appear essential for regulating the full activation of PLC- γ -mediated pathways required for capacitive Ca^{2+} entry in thymocytes.

In lymphocytes, data suggest that PLC- γ also contributes to the activation of ERKs in response to antigen receptor stimulation by a protein kinase C (PKC)-mediated activation of Ras and Raf, in addition to the well established GRB-2-son of sevenless (SOS) pathway (48, 49). Despite the relative integrity of GRB-2-associated proteins in *itk*^{-/-} and *rlk*^{-/-itk}^{-/-} cells, we observe a defect in ERK activation. Thus, this defective ERK activation may result from defects in a PLC-PKC-dependent pathway. It is notable that we do not observe defect in activation of p38 in these cells, despite defects in negative selection. Thus, p38 activation may be independent of PLC- γ activation and probably does not contribute to the defects in negative selection observed in *rlk*^{-/-itk}^{-/-} mice.

Our observed defect in ERK activation is particularly intriguing given the decreased CD4/CD8 ratio observed in *itk*^{-/-} and *rlk*^{-/-itk}^{-/-} mice. The factors that affect lineage decision commitment between CD4 and CD8 cells remain controversial (50, 51). Again, at least one model suggests that alterations in signaling intensity can help determine this lineage determination, with development of CD4⁺ cells requiring a stronger signal. However, several specific molecules have also been implicated in the regulation of this cell fate decision, including Notch (52), Lck (53, 54), and the MAPKs ERK 1 and 2 (28, 29). It is possible that these molecules may contribute to lineage determination by influencing signal intensity or duration or by permitting survival of cells that would normally die due to low signaling. Our data is consistent with that from studies by Bomhardt et al (28) and Sharp et al. (29), who demonstrate that decreased activation of ERKs can alter the lineage decision between CD4⁺ and CD8⁺ cells. As both *itk*^{-/-} and the *rlk*^{-/-itk}^{-/-} cells exhibit decreased activation of ERK 1 and 2 as a consequence of their defects in signaling downstream from the TCR (Fig. 7), the decreased CD4⁺ cell number and relative increase in CD8⁺ cells in both these strains is consistent with previous results using either MEKK inhibitors or a hypersensitive mutant of ERK 2. These data suggest that diminished signaling from the TCR can influence lineage commitment, perhaps by affecting activation of specific downstream molecules, and directly implicate Tec kinases as part of the signaling pathways influencing this decision.

These studies provide further insight into the biochemical intermediates that influence cell fate decisions in the thymus and contribute to the development of the T cell repertoire. Specifically, these data provide evidence that the Tec kinases contribute to setting thresholds required for

thymic selection and lineage commitment by controlling activation of downstream signaling molecules. Thus, proper activation of Tec family kinases may be critical for thymocyte selection decisions. In particular, the switch from negative selection to positive selection and the increased thymic cellularity in the *rlk*^{-/-itk}^{-/-} animals highlights the complex nature of thymic selection, where reduction of TCR signaling may not merely decrease selection but may alter selection and lineage decisions. How defects in these signaling pathways influence these developmental selection decisions may be further delineated through the generation of knockout mice deficient in all three Tec kinases expressed in T cells and the use of different TCR-transgenic mice with varying affinities and avidities of TCR engagement.

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