Restricting Zap70 Expression to CD4^{+}CD8^{+} Thymocytes Reveals a T Cell Receptor–dependent Proofreading Mechanism Controlling the Completion of Positive Selection

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Abstract

Although T cell receptor (TCR) signals are essential for intrathymic T cell–positive selection, it remains controversial whether they only serve to initiate this process, or whether they are required throughout to promote thymocyte differentiation and survival. To address this issue, we have devised a novel approach to interfere with thymocyte TCR signaling in a developmental stage-specific manner in vivo. We have reconstituted mice deficient for Zap70, a tyrosine kinase required for TCR signaling and normally expressed throughout T cell development, with a Zap70 transgene driven by the adenosine deaminase (ADA) gene enhancer, which is active in CD4^{+}CD8^{+} thymocytes but inactive in CD4^{+} or CD8^{+} single-positive (SP) thymocytes. In such mice, termination of Zap70 expression impaired TCR signal transduction and arrested thymocyte development after the initiation, but before the completion, of positive selection. Arrested thymocytes had terminated Rag gene expression and up-regulated TCR and Bcl-2 expression, but failed to differentiate into mature CD4 or CD8 SP thymocytes, to be rescued from death by neglect or to sustain interleukin 7Rα expression. These observations identify a TCR-dependent proofreading mechanism that verifies thymocyte TCR specificity and differentiation choices before the completion of positive selection.

Key words: T cell development • thymus • antigen receptor rearrangement • transgenic mice • adenosine deaminase

Introduction

The differentiation of CD4^{+}CD8^{+} double-positive (DP)* thymocytes into CD4^{+}CD8^{−} or CD4^{−}CD8^{+} single-positive (SP) thymocytes is a critical step during αβ T cell intrathymic development (1). DP thymocytes are short-lived cells that have successively rearranged their TCRβ genes, actively rearrange their TCRα genes, and express surface TCRαβ complexes upon productive TCRα rearrangement. Current models of T cell development propose that the fate of DP thymocytes is dictated by their TCR avidity for self-MHC peptide complexes (MHC-p) expressed on the thymic stroma (2–4), so that only those DP thymocytes whose TCR binds self MHC-p with intermediate avidity differentiate into mature T cells, a process referred to as positive selection which requires intracellular TCR signaling (5, 6).

Thymocytes undergoing positive selection are subject to multiple developmental changes, including the cessation of Rag-1 and Rag-2 recombinase gene expression (7, 8), which terminates TCRα gene rearrangement and fixes TCR specificity, the up-regulation of Bcl-2 and of surface TCR expression (4, 9, 10), the termination of CD8 or CD4 expression (3, 4), and the acquisition of the IL-7–dependent survival pattern that characterizes mature T cells (11–15). While TCR signaling is required for these differentiation events, how TCR signals promote them remains unclear. As these differentiation events occur sequentially rather than simultaneously, it can be envi-

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Abbreviations used in this paper: ADA, adenosine deaminase; DP, double positive; MFI, mean fluorescence intensity; MHC-p, MHC-peptide complex; SP, single positive.

sioned that TCR signals only serve at the onset of the selection process, to initiate in DP thymocytes a developmental sequence which then progresses in a TCR-independent manner. Alternatively, it is possible that persistent or repeated TCR signaling is required throughout positive selection.

To examine this issue, it is necessary to disrupt TCR engagements or TCR signal transduction in thymocytes after the initiation, but before the completion, of positive selection. Achieving this goal directly in vivo has so far remained elusive, and indirect attempts based on adoptive cell transfer or in vitro signaling strategies have led to conflicting conclusions: while some observations suggest that multiple TCR signaling events are required for positive selection (16, 17), others support the opposite view, namely that a short-duration TCR signaling episode 'commits' DP thymocytes to a differentiation sequence that is subsequently TCR independent (18).

The objective of the present study was to address these issues in vivo, by limiting the developmental window of TCR signal transduction in thymocytes. Our approach was to confine to DP thymocytes the expression of Zap70, a tyrosine kinase normally expressed throughout T cell development (19, 20). This choice was driven by the fact that Zap70 gene disruption precludes TCR signal transduction in DP thymocytes but does not affect their generation: Zap70° mice have no SP thymocytes nor mature T cells, but normal numbers of TCRαβ-expressing DP thymocytes (5, 6).

Here we reconstituted Zap70° mice with a Zap70 transgene expressed in DP but not in SP thymocytes, thereby selectively enabling TCR signal transduction in DP thymocytes. As a result, intrathymically signaled thymocytes initiated, but failed to complete, positive selection, demonstrating that positive selection requires repeated or persistent TCR signals beyond the DP stage. However, while 'DP-restricted' TCR signals failed to reconstitute positive selection, they promoted its initial steps, including the fixation of TCR specificity and the up-regulation of TCR and Bcl-2 expression. These findings reveal a late proofreading TCR signaling step during positive selection and provide a novel approach to dissect this process.

Materials and Methods

Generation of Transgenic Mice. A cDNA encoding a C-terminally myc-tagged version of mouse Zap70 was generated by oligonucleotide-directed mutagenesis of the murine Zap70 cDNA (21). The predicted C-terminal amino acid sequence of the resulting protein was as follows (single letter code, a slash separates Zap70 and myc sequences): vaeaacg/ELASMEQKLISEEDLNNG. A human adenosine deaminase (ADA)-based transgenic expression vector was constructed from plasmid pADACAT 4/12 (22) by replacing chloramphenicol acetyl transferase sequences with the myc-tagged Zap70 cDNA. Detailed cloning procedures are available on request. The DNA sequence of all oligonucleotide-encoded regions was verified by dideoxy sequencing. The transgene DNA was microinjected in fertilized C57BL/6 (B6) oocytes as described previously (23). Founder mice were identi-

fied by Southern blotting on tail DNA; transgenic mice were backcrossed to Zap70° mice and subsequently identified by PCR on tail DNA. Both myc-tagged and untagged Zap70 cDNAs were also inserted into pcDNA3 (Invitrogen) for expression in P116 cells.

Animals. Wild-type (B6) mice were from the National Cancer Institute (NCI) animal facility. Zap-70° mice (5) were obtained from Dr. Alfred Singer (NCI); mice transgenic for the P14 TCR (24) on an H-2^b background were from The Jackson Laboratory. Except where otherwise indicated, mice were analyzed between 5 and 10 wk of age, and were heterozygous for the transgene(s) they carry. All mice used in this study were housed and cared for in accordance with National Institutes of Health guidelines.

Antibodies. The following mAbs were used for staining: anti c-myc (9E10; Santa Cruz Biotechnology, Inc.); anti-TCRβ (H57–597), anti-CD4 (RM4.4 and GK1.5), anti-CD8 (53–6.7), anti-CD69 (H1.2F3), anti-HSA (CD24, M1/69), anti–IL-7Rα, and anti–mouse Bcl-2 (3F11), all from BD Biosciences. The following antibodies were used for immunoprecipitation or immunoblotting: anti-phosphotyrosine (4G10; Upstate Biotechnology), anti-phospho-Erk (E10) and anti-Erk2 (both from Cell Signaling Technologies), anti-LAT (25), and anti-Zap70 (both from Dr. Lawrence Samelson, NCI).

Cell Preparation, Staining, and Stimulation. Single cell suspensions of thymocytes, splenocytes, or LN cells were prepared and surface stained as described previously (10). Splenocytes suspensions were cleared of red blood cells by ACK treatment. Cells were stained for intracellular transgenic Zap70 (9E10 anti-myc) or Bcl-2 after 10 min fixation (2% formaldehyde PBS, room temperature) and 5 min permeabilization in IC staining buffer (0.03% saponin, 0.1% BSA, 0.1% NaN$_3$ PBS). Intracellular staining for detection of phospho-Erk molecules was performed after 3 min fixation (8% formaldehyde PBS, room temperature) and permeabilization in IC staining buffer. Cell fluorescence was measured, typically on 10^5 cells, on a 2-laser FACSCalibur™ (BD Biosciences) with 4-decade logarithmic amplification, and analyzed using FlowJo software. Live cells were identified by forward light scatter and propidium iodide gating, or by forward and side light scatter gating for 4-color and intracellular staining analyses. Purified thymocyte populations were prepared using anti-CD4 (GK1.5), anti-CD8 (53–6.7), or anti–FITC coated magnetic beads (Miltenyi Biotec) as follows: (a) DP thymocytes by selection of CD4$^+$ cells (P14 TCR transgenic mice), or by selection of CD8$^+$ cells (all other mice), (b) CD69$^+$ cells by selection of FITC$^+$ cells after cell surface staining with FITC-conjugated anti-CD69. Cell purity, assessed by surface staining and flow cytometry, was >95% except where otherwise indicated. LN T cells (cell purity >90%) were B cell depleted using anti–mouse immunoglobulin magnetic beads (Polysciences). For in vitro stimulation of DP thymocytes, cells were coated for 10 min at 4°C with biotinylated anti-TCRβ and anti-CD4 (GK1.5), both from BD Biosciences, cross-linked with streptavidin (Southern Biotechnology Associates, Inc.), and processed for immunoprecipitation and immunoblotting as described (23). P116 cells were transfected by electroporation, distributed on plates previously coated with anti-CD3 (UCHT-1; BD Biosciences) at the indicated concentration, and analyzed for CD69 expression after 16 h at 37°C.

RT-PCR. Total RNA was extracted using Trizol (Invitrogen) and reverse-transcribed by oligo-dT priming using the Thermoscript RT-PCR Kit (Invitrogen). Taq-mediated PCR was performed for 35 cycles using the following parameters for

each cycle: 94 °C (45 s), 55 °C (45 s), and 72 °C (2.5 min). PCR products were separated by agarose gel electrophoresis and visualized by ethydium bromide staining. PCR primers were as follows: Rag-1 (GenBank XM_123916) (5′ccaagctgcagacattctagcactc3′ and 5′caacatctgccttcacgtcgatcc3′); Rag-2 (GenBank NM_009020) (5′cacatccacaagcaggaagtacac3′ and 5′ggttcagggacatctcctactaag3′) and β-actin (GenBank AF054837) (5′gtgggccgctctaggcacc3′ and 5′ctctttgatgtcacgcacgat3′).

Calcium Flux Measurements. Calcium fluxes were measured following published procedures (26). Briefly, thymocytes were loaded with 1.8 μM indo-1 AM (Molecular Probes) for 30 min at 37°C, washed twice, incubated for 30 min at 4°C with biotinylated anti-TCRβ, or with anti-TCRβ and anti-CD4 (GK 1.5), and with fluorochrome-conjugated mAb against CD8 and CD4 (RM4.4, which does not cross react with GK1.5), and finally washed twice. Cell stimulation (at 37°C) and fluorescence measurements were performed on a specially equipped FACStar[PLUS™] (BD Biosciences). TCR cross-linking was initiated 2 min after transferring cells at 37°C by adding 2 μg/ml streptavidin. Fluorescence was collected over 512 s and analyzed using FlowJo software.

Results

A Mouse Line Expressing Zap70 in DP but Not in SP Thymocytes. To confine TCR signaling to DP thymocytes, we reconstituted Zap70° mice with a Zap70 transgene driven by a human ADA DP-specific enhancer, active in DP thymocytes but not in SP thymocytes or in mature T cells (22; Fig. 1 A). Transgenic Zap70 molecules were appended a C-terminal myc epitope tag to facilitate their detection. Transient transfection analyses in Zap70-deficient P116 Jurkat cells (27) verified that the myc tag did not impair Zap70 function (Fig. 1 B). Two independent mouse lines, referred to as A and D2, expressed this transgene in DP thymocytes with little or no residual expression in SP thymocytes or mature T cells. As both lines generated similar phenotypes after backcrossing to Zap70° mice, and as transgene expression was 1.5–2 fold higher in line A than in line D2 thymocytes (unpublished data), we report here analyses made with line A mice.

We assessed transgene expression by immunoblotting Zap70 molecules immunoprecipitated from cells heterozygous for endogenous Zap70 and for the ADA-Zap70 transgene (Zap70^{+/−}ADA-Zap70^{+/−}). Transgenic Zap70 molecules, which migrate with reduced electrophoretic mobility because of the epitope tag, were detected in DP thymocytes but not in CD4 and CD8 SP thymocytes or in lymph node T cells (Fig. 1 C); in DP thymocytes, the ratio of transgenic to endogenous Zap70 molecules was 0.47:1, as assessed by densitometric scanning and serial dilution analyses (Fig. 1 C, and unpublished data). As a result, Zap70 expression in Zap70° thymocytes homozygous for the ADA-Zap70 transgene was similar (98 ± 13% based on three distinct experiments) to that in Zap70^{+/−} thymocytes (Fig. 1 C, lanes 6 and 8). Confirming these results, analyses of transgene expression by anti–myc-epitope intracellular staining detected transgenic Zap70 in DP thy-
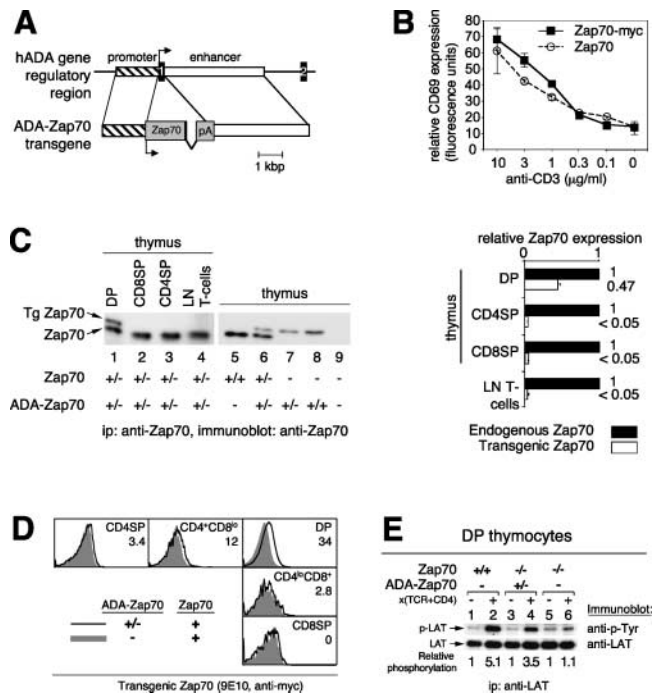

Figure 1. Expression of the ADA-Zap70 transgene. (A) Schematic of the ADA-Zap70 transgene. A C-terminally myc-tagged murine Zap70 cDNA was inserted between the promoter and the first intron enhancer of the human ADA gene. Numbered boxes depict the first and second exon of the ADA gene, pA indicates intronic and polyadenylation sequences from the polyoma middle T gene and black arrows the transcription start sites. (B) myc-epitope tagged Zap70 molecules are fully functional. Zap70-deficient P116 cells were transiently transfected with myc-tagged or untagged Zap70 expression vectors, or with an empty expression vector (mock). Dose–response curves to TCR stimulation were established by measuring anti-CD3-induced CD69 expression; for each antibody concentration, CD69 fluorescence is expressed as ([MFI of transfected cells] − [MFI of mock-transfected cells]) where MFI is the mean fluorescence intensity. (C) The ADA-Zap70 transgene is expressed in DP thymocytes but not in SP thymocytes and peripheral T cells. (left) Purified DP, CD8SP, and CD4SP thymocytes, and LN T cells (lanes 1–4) or unseparated (>85% DP) thymocytes (lanes 5–9), were lysed in 1% Triton X-100 buffer and assessed for endogenous and transgenic Zap70 by anti-Zap70 immunoprecipitation and immunoblotting; (right) expression of transgenic Zap70 relative to that of endogenous Zap70 in each cell population of Zap70^{+/−} mice heterozygous for the ADA-Zap70 transgene was quantified by autoradiogram scanning of three distinct experiments. Also note that endogenous Zap70 expression was 1.4-fold higher in Zap70^{+/+} than in Zap70^{+/−} thymocytes (lanes 5 and 6). (D) Flow cytometric analysis of transgenic Zap70 expression. Thymocytes from wild-type and transgenic mice were stained for surface CD4 and CD8 and for intracellular transgenic Zap70 (9E10 anti-myc), and analyzed by flow cytometry. Numbers within each box indicate transgenic Zap70 expression, calculated in each population as: 100 × ([MFI of transgenic cells] − [MFI of nontransgenic cells])/(MFI of nontransgenic cells). Results are the mean from three independent experiments. (E) Activity of Zap70 transgenic Zap70 molecules. Purified DP thymocytes were stimulated for 5 min by anti-TCR + anti-CD4 cocrosslinking, lysed, and assessed for LAT tyrosine phosphorylation by immunoblotting of anti-LAT immunoprecipitates. Equal loading of the lanes was verified by anti-LAT immunoblotting. The relative stimulation of LAT phosphorylation is indicated for each mouse genotype.

mocytes but not significantly in CD4 or CD8 SP thymocytes (Fig. 1 D). Thus, the ADA-Zap70 transgene is expressed in DP thymocytes, but not in SP thymocytes or mature T cells.

The ADA-Zap70 Transgene Fails to Restore Positive Selection. We crossed ADA-Zap70 transgenic and Zap70° mice to generate Zap70° mice heterozygous for the ADA-Zap70 transgene (Zap70°ADA-Zap70$^{+/-}$, thereafter referred to as ADA-ZapA). That the transgenic protein was active in DP thymocytes was confirmed by its ability to promote TCR + CD4-induced tyrosine-phosphorylation of LAT, a direct substrate of Zap70 (25), in ADA-ZapA DP thymocytes (Fig. 1 E, lanes 3–6). To examine if the ADA-Zap70 transgene restored positive selection in the absence of endogenous Zap70, we assessed thymocyte and T cell populations in ADA-ZapA mice by surface staining and flow cytometry. Normally, DP thymocytes undergoing positive selection up-regulate TCR surface expression as they differentiate into CD4 or CD8 SP cells, and subsequently down-regulate HSA (CD24) surface expression (28); thus, the number of HSA^{lo} SP thymocytes is indicative of the efficiency of positive selection. Importantly, ADA-ZapA mice had essentially no HSA^{lo} CD4 or CD8 SP thymocytes, indicating that the transgene failed to restore positive selection in mice lacking endogenous Zap70 (Fig. 2 A). Consistent with this conclusion, peripheral T cell populations were absent in newborn and 2-wk-old ADA-ZapA mice (unpublished data) and remained marginal in young adults (Fig. 2 A), possibly arising by peripheral expansion of infrequent thymus-derived T cells that overcame the intrathymic differentiation block. However, ADA-ZapA thymi critically differed from Zap70° thymi in that some ADA-ZapA thymocytes were intrathymically signaled to initiate selection, as evidenced by their TCR^{hi} DP or transitional (CD4$^+$CD8^{lo} or CD4^{lo}CD8$^+$) surface phenotype (Fig. 2 B and Table I). Thus, while the T cell development block was essentially as complete in ADA-ZapA and Zap70° mice, the block in ADA-ZapA mice occurred at a subsequent developmental stage, namely in CD4$^+$CD8^{lo} and CD4^{lo}CD8$^+$ thymocytes which are developmental intermediates between DP and SP thymocytes (29–31).

Strikingly, the same differentiation block was observed in Zap70° mice homozygous for the ADA-Zap70 transgene (ADA-ZapA$^{+/+}$; Fig. 2, A and B), despite the fact that ADA-ZapA$^{+/+}$ DP thymocytes expressed as much Zap70 as Zap70$^{+/-}$ DP thymocytes which differentiated normally into SP cells (Fig. 1 C). While both ADA-ZapA and ADA-ZapA$^{+/+}$ thymi had significant, albeit reduced, numbers of CD4$^-$CD8$^+$ thymocytes, these CD4$^-$CD8$^+$ cells remained HSA^{hi} and failed to give rise to normal CD8 T cell populations. These observations demonstrate that the cessation of Zap70 expression during the DP to SP transition prevents the completion of intrathymic T cell differentiation.

The ADA-Zap70 Transgene Enables TCR Signaling in DP but Not in Transitional Thymocytes. Given the unusual developmental block exhibited by the ADA-ZapA mouse, we examined in more detail how the ADA-Zap70 transgene restored TCR signal transduction, by assessing in ADA-ZapA thymocytes two critical targets of TCR signaling during positive selection (32–34): calcium mobilization, measured by flow cytometric analysis of calcium-dependent indo-1 fluorescence, and MAP kinase activa-
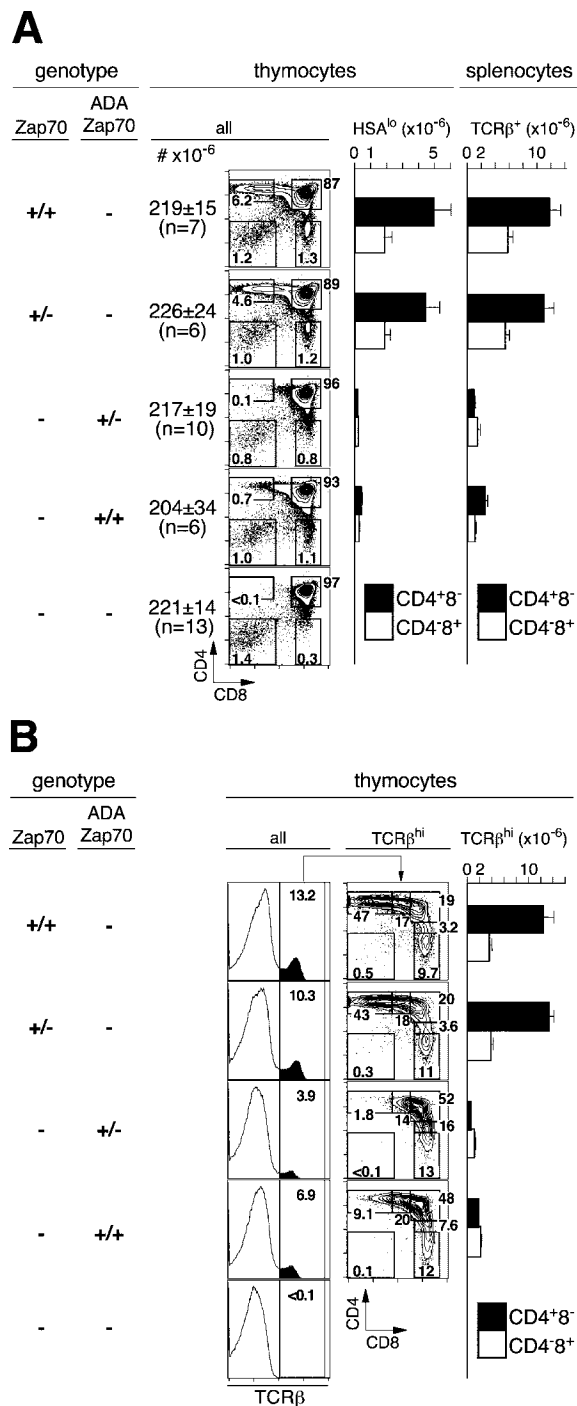

Figure 2. Expression of the ADA-Zap70 transgene in Zap70° mice fails to restore positive selection. Thymocyte and splenocyte suspensions were enumerated by trypan blue exclusion (total cell numbers are shown as mean ± SEM) and assessed by surface staining and flow cytometry for expression of CD4, CD8, and HSA (A, thymocytes) or CD4, CD8 and TCRβ (A, splenocytes; B). Populations were quantified by multiplying total cell numbers by the fraction of cells in that population and are shown as mean ± SEM. Numbers next to or within boxes (A and B, two color contour plots) indicate the percentage of cells in that box. Note that CD4$^{-/lo}$CD8$^+$ cells in Zap70° mice were TCR^{lo} precursors of DP thymocytes which were also present in all other strains.

Table I. *TCR^{hi} Transitional Cells Do Not Accumulate with Age in ADA-ZapA Thymi*

		Cell numbers ($\times 10^{-6}$)[a]		
Age	Genotype	TCR^{hi} DP	TCR^{hi}CD4$^+$CD8^{lo}	TCR^{hi}CD4^{lo}CD8$^+$
4 d	Zap70$^{+/+}$	0.19 ± 0.01	0.08 ± 0.01	0.05 ± 0.003
	ADA-ZapA	0.17 ± 0.005	0.02 ± 0.005	0.07 ± 0.004
	Zap70°	<0.01	<0.001	<0.001
2 wk	Zap70$^{+/+}$	0.98 ± 0.11	0.51 ± 0.05	0.11 ± 0.01
	ADA-ZapA	0.8	0.28	0.17
	Zap70°	<0.01	<0.01	<0.01
6–8 wk	Zap70$^{+/+}$	5.1 ± 0.48	3.8 ± 0.57	1.0 ± 0.16
	ADA-ZapA	6.2 ± 0.74	1.9 ± 0.17	2.0 ± 0.33
	Zap70°	0.23 ± 0.04	0.03 ± 0.005	0.04 ± 0.001

[a]Thymocytes were triple stained for CD4, CD8, and TCRβ surface expression. Cell numbers were calculated as in the legend to Fig. 2 and are indicated as mean ± SEM. At least two mice of each genotype were analyzed for each time point, with the exception of 2-wk-old mice, in which two Zap70$^{+/+}$ and one ADA-ZapA mice were analyzed.

tion, measured by intracellular staining and by immunoblot analyses, using a monoclonal antibody which recognizes dually (threonine/tyrosine) phosphorylated, activated, Erk1 and Erk2 molecules (35).

TCR + CD4-induced calcium mobilization (Fig. 3 A, top) and Erk phosphorylation (Fig. 3 C, top, and Fig. 3 D, lanes 3–6) were both disrupted in Zap70° DP thymocytes, and restored by the ADA-Zap70 transgene. Of note, while heterozygous expression of the transgene was sufficient to restore TCR + CD4-induced Erk phosphorylation (Fig. 3 D), calcium mobilization appeared to be more stringently dependent on Zap70 levels, as TCR + CD4-induced calcium fluxes increased with Zap70 expression (Fig. 3 A, compare the progression from ADA-ZapA to Zap70$^{+/+}$). Unlike in DP thymocytes, TCR + CD4-induced calcium mobilization and Erk phosphorylation were both markedly impaired in ADA-ZapA CD4$^+$CD8^{lo} thymocytes relative to their Zap70$^+$ counterparts (Fig. 3, A and C, bottom). Furthermore, cross-linking of TCR alone, which allowed simultaneous assessment of CD4$^+$CD8^{lo} and CD4^{lo}CD8$^+$ thymocyte responses by providing a coreceptor-independent stimulation, induced strong calcium responses in wild-type CD4$^+$CD8^{lo} and CD4^{lo}CD8$^+$ populations, but failed to do so in their ADA-ZapA counterparts (Fig. 3 B). Thus, as assessed on MAP kinase activation and calcium mobilization, the ADA-Zap70 transgene reconstituted Zap70° mice for TCR signal transduction in DP thymocytes but not in CD4$^+$CD8^{lo} and CD4^{lo}CD8$^+$ transitional thymocytes; of note, these observations demonstrate that TCR signaling requires Zap70 beyond the DP stage of T cell development in mice.

Two Genetically Separable Signaling Steps during Positive Selection. We next examined if intrathymically signaled ADA-ZapA DP thymocytes normally initiated their differ-entiation. We first documented that normal numbers of ADA-ZapA DP thymocytes expressed CD69, one of the earliest marker of positive selection (36, 37), in contrast to Zap70° DP thymocytes which all were CD69$^-$ (Fig. 4 A). Second, we showed that CD69$^+$ ADA-ZapA thymocytes had been signaled to terminate Rag-1 and Rag-2 gene expression, a critical event during positive selection (7, 8, 38–41): Rag-1 and Rag-2 expression was reduced by 10-fold in intrathymically signaled ADA-ZapA CD69$^+$ cells relative to their CD69$^-$ counterpart (Fig. 4 B). Finally, we assessed ADA-ZapA DP thymocytes for expression of antiapoptotic Bcl-2 molecules and of the IL-7 receptor α-subunit (IL-7Rα), which is necessary to naive T cell survival after their exit from the thymus (11, 12, 14, 15). Bcl-2 and IL-7Rα, which are silent on most DP thymocytes (9, 10, 13), were up-regulated by TCR^{hi} DP thymocytes in both Zap70$^+$ and ADA-ZapA mice (Fig. 4 C). These observations indicated that ADA-ZapA DP thymocytes normally initiated their differentiation.

In contrast, a series of observations indicated that the further development of ADA-ZapA transitional thymocytes was markedly affected by the cessation of Zap70 expression. First, ADA-ZapA transitional thymocytes failed not only to progress to the SP stage, but also to accumulate over time, indicating that they were not rescued from death by neglect: the total number of ADA-ZapA transitional thymocytes was less than that in age-matched Zap70$^+$ mice, at ages 4 d, 2 wk, and 6–8 wk (Fig. 5 A). Furthermore, IL-7Rα and CD69 levels were lower on ADA-ZapA than on Zap70$^+$ transitional thymocytes, a difference modest on CD4$^+$CD8^{lo} cells but particularly striking on CD4^{lo}CD8$^+$ thymocytes (Fig. 5 B, a). Importantly, the same was true of ADA-ZapA$^{+/+}$ transitional thymocytes and of the small population of TCR^{hi}CD4$^-$CD8$^+$
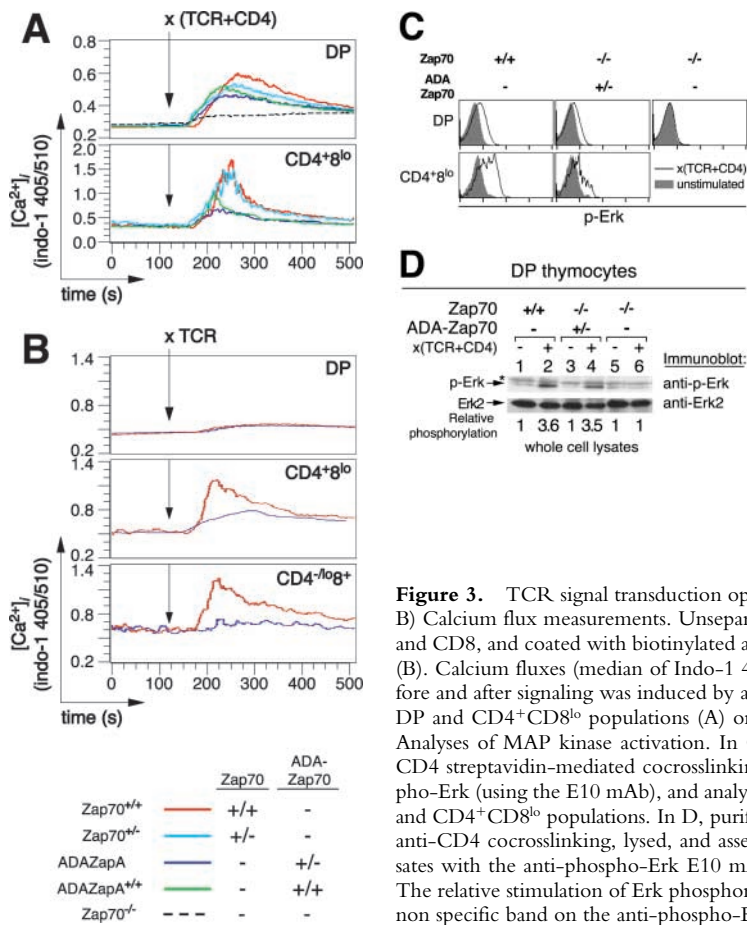
Figure 3. TCR signal transduction operates in DP but not in transitional ADA-ZapA thymocytes. (A and B) Calcium flux measurements. Unseparated thymocytes were loaded with indo-1, surface stained for CD4 and CD8, and coated with biotinylated anti-TCRβ and anti-CD4 (A) or with biotinylated anti-TCRβ only (B). Calcium fluxes (median of Indo-1 405/Indo-1 510 fluorescence ratio, vertical axes) were recorded before and after signaling was induced by addition of streptavidin at t = 120 s (arrow) and are shown for gated DP and CD4+CD8lo populations (A) or DP, CD4+CD8lo and CD4−/loCD8+ populations (B). (C and D) Analyses of MAP kinase activation. In C, unseparated thymocytes were stimulated by anti-TCR + anti-CD4 streptavidin–mediated cocrosslinking, fixed, stained for surface CD4 and CD8 and intracellular phospho-Erk (using the E10 mAb), and analyzed by flow cytometry; phospho-Erk staining is shown on gated DP and CD4+CD8lo populations. In D, purified CD8+ thymocytes (>95% DP) were stimulated by anti-TCR + anti-CD4 cocrosslinking, lysed, and assessed for Erk phosphorylation by immunoblotting of whole cell lysates with the anti-phospho-Erk E10 mAb. Equal lane loading was verified by anti-Erk2 immunoblotting. The relative stimulation of Erk phosphorylation is indicated for each mouse genotype. An asterisk indicates a non specific band on the anti-phospho-Erk blot.

thymocytes present in ADA-ZapA+/+ mice (Fig. 5 B, b, and unpublished data). Of note, Bcl-2 levels remained high in ADA-ZapA transitional thymocytes (Fig. 5 B), perhaps because cells with insufficient Bcl-2 expression were rapidly eliminated from the thymus by programmed cell death. Altogether, these observations demonstrate that the cessation of TCR signaling in transitional thymocytes disrupted the completion of thymocyte differentiation.
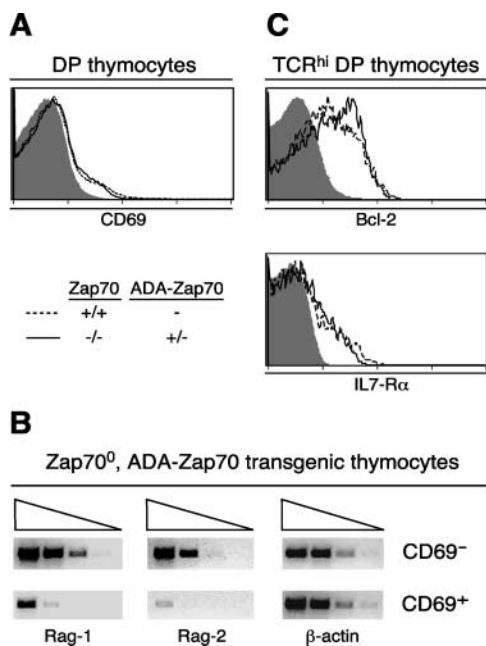

Figure 4. Intrathymic TCR signaling promotes the differentiation of ADA-ZapA DP thymocytes. (A) Thymocytes were stained for CD4, CD8, and CD69. CD69 levels on gated DP thymocytes are shown as single-color histograms for each mouse genotype. (B) Intrathymically signaled (CD69+) ADA-ZapA thymocytes down-regulate Rag gene expression. CD69+ and unsignaled CD69− ADA-ZapA thymocytes were separated with magnetic beads. The CD69+ population was 85% CD69+ (of which 80% were CD4+CD8+, and 20% CD4+CD8lo or CD4loCD8+) and 15% CD69−/lo (all CD4+CD8+; not shown). RT-PCR analysis showed a 10-fold decreased Rag-1 and Rag-2 gene expression in CD69+ (bottom) compared with CD69− (top) cells, corresponding to the degree of purity of the CD69+ population. For each cDNA preparation, PCRs were performed from 300, 100, 30, and 10 cell equivalents (left to right). β-actin expression, assessed in parallel as a control for cDNA preparation, was identical in both populations. (C) Zap70+/+ and ADA-ZapA thymocytes were stained for surface TCRβ, CD4, and CD8, and for intracellular Bcl-2 (top) or for surface IL-7Rα, TCRβ, CD4, and CD8 (bottom). Surface IL-7Rα and intracellular Bcl-2 levels on gated TCRhi DP thymocytes are shown as single color histograms, and were identical in ADA-ZapA and Zap70+/+ cells. For comparison, shaded histograms show Bcl-2 (top) or IL-7Rα (bottom) levels on TCRβlo Zap70+/+ DP thymocytes.
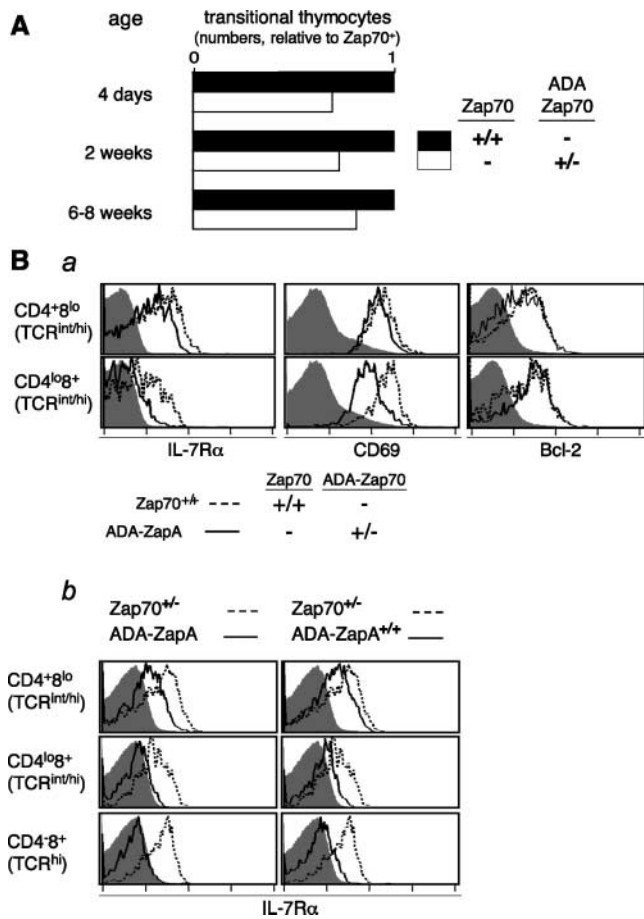
Figure 5. ADA-ZapA transitional thymocytes fail to differentiate. (A) Transitional thymocytes do not accumulate with age in ADA-ZapA thymi. Total numbers of both TCR^{hi}CD4$^+$CD8^{lo} and TCR^{hi} CD4^{lo}CD8$^+$ transitional thymocytes were calculated (from data shown in Table I) in ADA-ZapA and Zap70$^{+/+}$ mice aged 4 d, 2 wk, and 6–8 wk. Numbers of transitional thymocytes in ADA-ZapA mice (white bars) are expressed relative to those in their aged-matched Zap70$^{+/+}$ counterparts (set to 1 for each age group, black bars). (B) Transitional thymocytes fail to sustain CD69 and IL-7Rα expression in the absence of TCR signals. (a) Zap70$^{+/+}$ and ADA-ZapA thymocytes were stained as in Fig. 4 C. CD69, IL-7Rα, and intracellular Bcl-2 levels on gated TCR^{hi} CD4$^+$ CD8^{lo} (top) or CD4^{lo}CD8$^+$ (bottom) thymocytes are shown as single color histograms. Expression of both CD69 and IL-7Rα, but not of Bcl-2, was lower in ADA-ZapA than in Zap70$^{+/+}$ cells. (b) IL-7Rα expression was assessed as in the legend to Fig. 4 C on TCR^{hi} transitional and CD4$^-$CD8$^+$ thymocytes from ADA-ZapA (plain lines, left) and ADA-ZapA$^{+/+}$ (plain lines, right) mice and compared with that on their Zap70$^{+/-}$ counterparts (dashed lines). For comparison, shaded histograms in panels a and b show CD69, IL-7Rα, or intracellular Bcl-2 levels on Zap70$^+$ DP thymocytes.

Finally, to verify that MHC-p ligands that normally promote positive selection initiate the differentiation of ADA-ZapA thymocytes, we generated ADA-ZapA mice in which all thymocytes express the MHC I–restricted P14 TCR transgene, which promotes positive selection of CD8 T cells in Zap70$^+$ H-2D^b mice (24). Unlike Zap70$^+$ thymocytes expressing the P14 transgene, ADA-ZapA thymocytes expressing the P14 transgene (ADA-ZapA/P14) failed to differentiate into CD8 SP cells (Fig. 6 A). How-
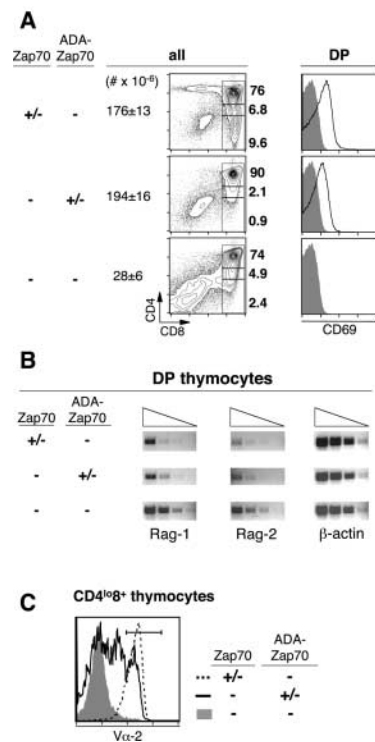
Figure 6. ADA-ZapA DP thymocytes initiate positive selection in response to normally positively selecting MHC-p. (A) Single cell thymocyte suspensions were prepared from mice of the indicated genotypes, all expressing the P14 TCR transgene. Thymocytes were assessed for CD4, CD8, and CD69 expression by 3-color flow cytometry. Left, 2-color contour plots show the absence of mature CD8SP cells in ADA-ZapA/P14 mice. Right, single parameter analyses of CD69 expression show up-regulated CD69 expression in P14-transgenic Zap70$^{+/+}$ and ADA-ZapA DP thymocytes (black lines), but not in Zap70$^°$ thymocytes (filled gray histograms). Note the previously described reduced thymic cellularity in TCR transgenic Zap70$^°$ mice (reference 42). One representative experiment is shown out of three. (B) Rag-1 and Rag-2 gene expression was analyzed on purified CD4$^+$ thymocytes (>95% DP) from the indicated mice. PCRs were performed from 300, 100, 30, and 10 cell-equivalents. (C) Thymocytes were assessed for CD4, CD8, TCRβ, and Vα-2 expression by 4-color flow cytometry. Single-color histograms show Vα-2 expression on CD4^{lo}CD8$^+$ populations. A bracket indicates the Vα-2^{hi} population; all such Vα-2^{hi} thymocytes were TCRβ^{hi}, whereas most Vα-2$^{-/lo}$ thymocytes were TCRβ^{lo}, and thus presumably pre-DP thymocytes.

ever, ADA-ZapA/P14 thymocytes had initiated positive selection, as indicated (a) by their elevated CD69 and reduced Rag-1 and Rag-2 expression relative to their Zap70$^°$ counterparts (Fig. 6 A, right and 6 B), and (b) by the fact that they differentiated into CD4^{lo}CD8$^+$ thymocytes expressing high levels of the transgenic Vα−2 chain (Fig. 6 C). We conclude from these observations that TCR/ MHC-p interactions that normally promote positive selection in Zap70$^+$ mice cause ADA-ZapA thymocytes to initiate positive selection. Unexpectedly, CD8 SP and CD4^{lo}CD8$^+$ thymocytes from P14 transgenic Zap70$^+$ mice only had marginal surface IL-7Rα expression (unpublished

data), preventing us from using this parameter to assess the differentiation status of CD4^{lo}CD8$^+$ cells from ADA-ZapA/P14 mice.

Discussion

In this study, our objective was to examine if thymocytes that have been TCR-signaled to initiate positive selection complete their development in a TCR-independent manner. To this end, we restricted the developmental window of TCR signaling by generating a novel mouse model in which Zap70 expression, hence TCR signal transduction, is confined to DP thymocytes. Using this approach, we show that thymocyte differentiation requires TCR signals throughout positive selection, that is, that thymocytes proofread their TCR specificity and differentiation choices before completing their intrathymic development.

Stage-specific Zap70 Expression as a New Model to Study Positive Selection. In the present study, we constructed a Zap70 transgene driven by a human ADA enhancer active in cortical (DP) but not medullary (SP) thymocytes (22), and showed that this transgene reconstitutes Zap70^o mice for both Zap70 expression and TCR signal transduction in DP, but not in CD4$^+$CD8^{lo} and CD4^{lo}CD8$^+$, thymocytes. Unlike the ADA-Zap70 transgene, Zap70 transgenes driven by the Lck promoter had previously been reported to restore positive selection in Zap70^o mice (5, 42). We think this difference is explained by the fact that the Lck proximal promoter is active in SP thymocytes (43), unlike the ADA enhancer used in the present study.

Importantly, the ADA-Zap70 transgene restored the initial steps of positive selection by all phenotypic criteria we assessed, including the termination of Rag expression and the up-regulation of CD5 (unpublished data), CD69, TCR, and Bcl-2; thus, ADA-ZapA and ADA-ZapA$^{+/+}$ thymocytes fail to differentiate into mature T cells not because they did not properly initiate positive selection, but because they become signaling-defective after they initiate positive selection and fail to generate late TCR signals required to complete their differentiation.

Persistent or Repeated TCR Signaling Proofreads Differentiation Decisions during Positive Selection. Our observations show that thymocyte survival and differentiation remain dependent on TCR signals after the fixation of TCR specificity, and the up-regulation of surface TCR and Bcl-2 expression, and thus demonstrate the existence of a late TCR signaling step that proofreads thymocyte differentiation choices during positive selection. The existence of a late 'rescue' or proofreading TCR signaling step required for the completion of positive selection has long remained controversial. That positive selection requires multiple TCR signaling steps was first suggested by the facts that it extends over several days in vivo (44) and cannot be reproduced by short-duration TCR engagements in vitro (45). However, assessment of this idea by adoptive cell transfer experiments gave rise to conflicting results. A study focused on positive selection by the HY transgenic TCR found that the development of HY transgenic T cells re-

quired selecting MHC molecules throughout positive selection (16). In contrast, a more extensive analysis of several TCR-transgenic and non–TCR-transgenic models reached the opposite conclusion, namely that intrathymically signaled (CD69$^+$) DP thymocytes need no additional MHC-p/TCR engagements to differentiate into SP cells (18). Finally, contradictory conclusions also emerged from recent studies in which DP thymocytes were signaled in vitro using pharmacological agents (46) or antigen-presenting cells (17).

The genetic strategy used in the present study clarifies these contradictions as it bypasses limitations inherent to indirect approaches. First, unlike adoptive cell transfers, the genetic strategy interrupts TCR signaling without disrupting MHC/TCR engagements and other potentially important thymocyte–stroma interactions (47). Another limitation of cell transfer approaches is that they necessarily rely on the assumption that the adoptively transferred 'signaled' (CD69$^+$ or TCR^{hi}) DP thymocytes are precursors of SP thymocytes; in fact, while CD69 or TCR up-regulation precedes CD4 or CD8 SP thymocyte differentiation (36, 37), precursor–progeny relationships between 'signaled' (CD69^{hi} or TCR^{hi}) DP thymocytes and mature SP thymocytes remain debated (30, 31, 48). Finally, our genetic strategy ensures that thymocytes are TCR signaled by thymic epithelial cells, rather than by pharmacological agents, antibody-mediated TCR cross-linking, or by dendritic cells which, unlike thymic epithelial cells, express costimulatory molecules previously shown to promote thymocyte death (17, 49).

A Genetic Dissection of Positive Selection. Our approach reveals two genetically separable signaling steps during positive selection that serve, the first one to engage DP thymocytes into the selection process, and the second one to promote IL-7Rα up-regulation and rescue from death by neglect in transitional and SP thymocytes. Importantly, neither cell transfer nor gene targeted disruption approaches had so far succeeded in dissociating differentiation events during positive selection. In fact, the developmental block in ADA-ZapA mice is unique as (a) it is complete, unlike the partial blocks in Vav- or Erk1-deficient mice, or in mice expressing a 'dominant negative' version of Lck, all of which allow reduced numbers of DP cells to initiate selection and to differentiate into mature T cells (34, 50–54) and (b) as it occurs after the initiation of positive selection, unlike the block at an 'unsignaled' DP stage in Zap70^o thymocytes (5, 6). Of note, our observations are consistent with the idea that TCR signaling thresholds increase during the terminal maturation of SP thymocytes (55–57) and do not exclude the existence of subsequent, TCR-independent, maturation stages during the terminal differentiation of CD4 or CD8 SP thymocytes (58).

That TCR signals are required for transitional thymocytes to sustain IL-7Rα expression has important biological consequences. Although IL-7 function during positive selection remains controversial (59, 60), the critical role of IL-7 for T cell survival makes IL-7Rα up-regulation an essential event during SP thymocyte differentiation

(12–15). We propose that the TCR–dependence of IL-7Rα expression during positive selection is an important proofreading mechanism that ensures that only thymocytes with appropriate TCR specificity and matching coreceptor expression will be allowed to durably participate in the peripheral T cell repertoire.

Whether, and how, CD4/CD8 lineage choice occurs in ADA-ZapA mice was not directly addressed by the present study. The presence in ADA-ZapA mice of a CD4$^{-/lo}$CD8$^+$ population, which normally includes CD8 cell precursors (30, 31), indicates that the cessation of transgenic Zap70 expression did not preclude CD8 lineage choice; in contrast, the presence of a CD4$^+$CD8^{lo} population is not informative with regard to lineage choice, as this population normally includes cells on their way to become CD4 or CD8 SP thymocytes (30, 31). We and others have proposed that the duration of TCR signals during positive selection determines CD4/CD8 lineage choice, with persistent signals promoting CD4 lineage choice and transient signals promoting CD8 lineage choice (60–62); future experiments will use ADA-ZapA mice to address this issue.

Finally, it is interesting to relate the proofreading mechanism revealed by the present study to observations suggesting that DP thymocytes sequentially rearrange their TCRα loci, and thus eliminate previously rearranged TCRα genes (63, 64). As termination of Rag gene expression is not immediate in cells undergoing positive selection (7, 39), these observations raised the possibility, illustrated in mice carrying a TCRβ transgene and a rearranged TCRα allele 'knocked-in' the endogenous TCRα locus (65, 66), that a selecting TCRα chain might be deleted by subsequent TCRα rearrangement. The present study now indicates that thymocytes that eliminate selecting TCRα specificities after initiating positive selection, thereby discontinuing expression of selecting TCR$\alpha\beta$ complexes, would fail to develop as mature T cells.

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