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

Xiaolong Liu, Anthony Adams, Kathryn F. Wildt, Bruce Aronow, Lionel Feigenbaum, and Rémy Bosselut

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 expression, but failed to differentiate into mature CD4 or CD8 SP thymocytes, to be rescued from death by neglect or to sustain interleukin 7R 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 T cell intrathymic development (1). DP thymocytes are short-lived cells that have successively rearranged their TCR genes, actively rearrange their TCR\(\gamma\) genes, and express surface TCR\(\alpha\beta\) complexes upon productive TCR\(\alpha\) 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 TCR\(\alpha\) 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-
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<sup>+</sup> mice have no SP thymocytes nor mature T cells, but normal numbers of TCRαβ-expressing DP thymocytes (5, 6).

Here we reconstituted Zap70<sup>+</sup> 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/ELASMEQKLISEED-H11034/H9251/H9252. 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 dyeoxy 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<sup>+</sup> 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<sup>+</sup> mice (5) were obtained from Dr. Alfred Singer (NCI); mice transgenic for the P14 TCR (24) on an H-2<sup>b</sup> 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 (H.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). Splenocyte 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<sub>3</sub>, 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<sup>5</sup> 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 Biotech) as follows: (a) DP thymocytes by selection of CD4<sup>+</sup> cells (P14 TCR transgenic mice), or by selection of CD8<sup>+</sup> cells (all other mice), (b) CD69<sup>+</sup> cells by selection of FITC<sup>+</sup> 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 cultured 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
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° mice 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 each 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 thymocytes 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° SP thymocytes is indicative of the efficiency of positive selection. Importantly, ADA-ZapA mice had essentially no HSA° 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 TCRhi DP or transitional (CD4+/CD8° or CD4°/CD8hi) 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+/CD8lo and CD4lo/CD8hi 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+/CD8lo thymocytes, these CD4+/CD8lo cells remained HSAhi 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 gene 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°/CD8hi cells in Zap70° mice were TCRβ° precursors of DP thymocytes which were also present in all other strains.
lanes 3–6) were both disrupted in Zap70 dependent on Zap70 levels, as TCR restore TCR heterozygous expression of the transgene was sufficient to and restored by the ADA-Zap70 transgene. Of note, while compare the progression from ADA-ZapA to Zap70 calcium fluxes increased with Zap70 expression (Fig. 3 A, 2 wk Zap70 impaired in ADA-ZapA CD4 mobilization and Erk phosphorylation were both markedly Unlike in DP thymocytes, TCR and CD4-induced calcium mobilization and Erk phosphorylation were both markedly impaired in ADA-ZapA CD4⁺CD8⁺ 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⁺ and CD4⁺CD8⁺ thymocyte responses by providing a coreceptor-independent stimulation, induced strong calcium responses in wild-type CD4⁺CD8⁺ and CD4⁺CD8⁺ populations, but failed to do so in their ADA-ZapA counterparts (Fig. 3 B). Thus, as assessed on MAPK kinase activation and calcium mobilization, the ADA-Zap70 transgene reconstituted Zap70⁺ mice for TCR signal transduction in DP thymocytes but not in CD4⁺CD8⁺ and CD4⁺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 differentiation. 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 anti-apoptotic 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⁺⁺ 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-7Ra and CD69 levels were lower on ADA-ZapA than on Zap70⁺ transitional thymocytes, a difference modest on CD4⁺CD8⁻ cells but particularly striking on CD4⁺CD8⁺ thymocytes (Fig. 5 B, a). Importantly, the same was true of ADA-ZapA⁺⁺ transitional thymocytes and of the small population of TCR⁺⁺CD4⁻CD8⁺

| Age     | Genotype | TCR⁺ DP | TCR⁺CD4⁺CD8⁻ | TCR⁺CD4⁺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 |

*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.
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 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+CD8+ populations (A) or DP, CD4+CD8lo and CD4loCD8+ populations (B). (C and D) Analyses of MAP kinase activation. In C, unseparated thymocytes were stimulated with 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+CD8+ populations. In D, purified CD8+ thymocytes (>95% DP) were stimulated with 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.

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 untagged 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 TCRβ+ 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 TCRβ+ 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-2Kb 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). However, 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 CD4loCD8+ 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 CD4loCD8+ thymocytes from P14 transgenic Zap70+ mice only had marginal surface IL-7Rα expression (unpublished

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 CD4+CD8–CD69+ and CD8+CD4–CD69+ 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– (top) or CD4loCD8+ (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 assayed 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.

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+CD8+ thymocytes and Yo-2 expression by 4-color flow cytometry. Single-color histograms show Yo-2 expression on CD4+CD8+ populations. A bracket indicates the Yo-2b population; all such Yo-2b thymocytes were TRCβb, whereas most Yo-2a thymocytes were TRCβa, and thus presumably pre-DP thymocytes.
The Journal of Experimental Medicine

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 required 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+) 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+ or TCR+) 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).
Zap70 expression did not preclude CD8 lineage choice; in cursors (30, 31), indicates that the cessation of transgenic ing a TCR observations raised the possibility, illustrated in mice carry-loci, and thus eliminate previously rearranged TCR experiments will use ADA-ZapA mice to address this issue.

Finally, it is interesting to relate the proofreading mecha-nism revealed by the present study to observations suggest-ing 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 TCRαβ complexes, would fail to develop as mature T cells.

We thank L. Samelson for anti-LAT and anti-Zap70 antisera; L. Sameelson and R. Abraham for P116 cells; A. Singer for Zap70 mice, for supporting the initial phases of this project, and for helpful discussions; S. Sharrow for advice with flow cytometry; X. Tai and Q. Yu for advice with cell purification procedures; and J. Ashwell, A. Bhandoola, B.J. Fowlkes, and A. Singer for critical reading of the manuscript.

Submitted: 26 September 2002
Revised: 20 December 2002
Accepted: 20 December 2002

References
1. Robey, E., and B.J. Fowlkes. 1994. Selective events in T cell development. Annu. Rev. Immunol. 12:675–705.
2. Jameson, S.C., K.A. Hoggquist, and M.J. Bevan. 1995. Positive selection of thymocytes. Annu. Rev. Immunol. 13:93–126.
3. Kaye, J., M.L. Hsu, M.E. Sauron, S.C. Jameson, N.R. Gas-coigne, and S.M. Hedrick. 1989. Selective development of CD4+ T cells in transgenic mice expressing a class II MHC-restricted antigen receptor. Nature. 341:746–749.
4. Teh, H.S., P. Kisielow, B. Scott, H. Kishi, Y. Uematsu, H. Bluthmann, and H. von Boehmer. 1988. Thymic major his-tocompatibility complex antigens and the alpha beta T-cell receptor determine the CD4/CD8 phenotype of T cells. Nature. 335:229–233.
5. Negishi, I., N. Motoyama, K. Nakayama, K. Nakayama, S. Senju, S. Hatakeyama, Q. Zhang, A.C. Chan, and D.Y. Loh. 1995. Essential role for ZAP-70 in both positive and negative selection of thymocytes. Nature. 376:435–438.
6. Kadlecck, T.A., N.S. van Oers, L. Lefrancos, S. Olson, D. Finlay, D.H. Chu, K. Connolly, N. Killeen, and A. Weiss. 1998. Differential requirements for ZAP-70 in TCR signaling and T cell development. J. Immunol. 161:4688–4694.
7. Brandle, D., C. Muller, T. Rulicke, H. Hengartner, and H. Pircher. 1992. Engagement of the T-cell receptor during positive selection in the thymus down-regulates RAG-1 expression. Proc. Natl. Acad. Sci. USA. 89:9529–9533.
8. Turka, L.A., D.G. Schatz, M.A. Oettinger, J.J. Chun, C. Gorka, K. Lee, W.T. McCormack, and C.B. Thompson. 1991. Thymocyte expression of RAG-1 and RAG-2: termination by T cell receptor cross-linking. Science. 253:778–781.
9. Linette, G.P., M.J. Grusby, S.M. Hedrick, T.H. Hansen, L.H. Glüncher, and S.J. Korsmeyer. 1994. Bcl-2 is upregulated at the CD4+ CD8+ stage during positive selection and promotes thymocyte differentiation at several control points. Immunity. 1:197–205.
10. Punt, J.A., H. Suzuki, L.G. Granger, S.O. Sharrow, and A. Singer. 1996. Lineage commitment in the thymus: only the most differentiated (TCRβhicl-2hi) subset of CD4+CD8+ thymocytes has selectively terminated CD4 or CD8 synthesis. J. Exp. Med. 184:2091–2099.
11. Rathmell, J.C., E.A. Farkash, W. Gao, and C.B. Thompson. 2001. IL-7 enhances the survival and maintains the size of naive T cells. J. Immunol. 167:6869–6876.
12. Schluns, K.S., W.C. Kieper, S.C. Jameson, and L. Lefrancos. 2000. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. Nat. Immunol. 1:426–432.
13. Sudo, T., S. Nishikawa, N. Ohno, N. Akiyama, M. Tamakoshi, H. Yoshida, and S. Nishikawa. 1993. Expression and function of the interleukin 7 receptor in murine lymphocytes. Proc. Natl. Acad. Sci. USA. 90:9125–9129.
14. Vivien, L., C. Benoist, and D. Mathis. 2001. T lymphocytes need IL-7 but not IL-4 or IL-6 to survive in vivo. Int. Immunol. 13:763–768.
15. Tan, J.T., E. Dudl, E. LeRoy, R. Murray, J. Sprent, K.I. Bluthmann, and H. von Boehmer. 1988. Thymic major histocompatibility complex antigens and the alpha beta T-cell receptor determine the CD4/CD8 phenotype of T cells. Nature. 335:229–233.
16. Yasutomo, K., B. Lucas, and R.N. Germain. 2000. TCR sig-naling for initiation and completion of thymocyte positive selection has distinct requirements for ligand quality and present-ing cell type. J. Immunol. 165:3015–3022.
17. Hare, K.J., E.J. Jenkinson, and G. Anderson. 1999. CD69 expression discriminates MHC-dependent and -independent stages of thymocyte positive selection. J. Immunol. 162:3978–3983.
18. Chan, A.C., M. Iwashima, C.W. Turck, and A. Weiss. 1992. ZAP-70: a 70 kD protein-tyrosine kinase that associates with the TCR zeta chain. Cell. 71:649–662.
20. Chan, A.C., N.S. van Oers, A. Tran, L. Turka, C.L. Law, J.C. Ryan, E.A. Clark, and A. Weiss. 1994. Differential expression of ZAP-70 and Syk protein tyrosine kinases, and the role of this family of protein tyrosine kinases in TCR signalling. J. Immunol. 152:4758–4766.

21. Gauen, L.K., Y. Zhu, F. Letourneau, Q. Hu, J.B. Bolen, L.A. Matis, R.D. Klaukas, and A.S. Shaw. 1994. Interactions of p59fyn and ZAP-70 with T-cell receptor activation motifs: defining the nature of a signalling motif. Mol. Cell. Biol. 14: 3729–3741.

22. Aronow, B., D. Lattier, R. Silbiger, M. Dusing, J. Hutton, G. Jones, J. Stock, J. McNeish, S. Potter, D. Witte, et al. 1989. Evidence for a complex regulatory array in the first intron of the human adenosine deaminase gene. Genes Dev. 3:1384–1400.

23. Bosselut, R., S. Kubo, T. Guinter, J.L. Kopacz, J.D. Altman, L. Feigenbaum, and A. Singer. 2000. Role of CD8beta domains in CD8 coreceptor function: importance for MHC I binding, signaling, and positive selection of CD8+ T cells in the thymus. Immunity. 12:409–418.

24. Ohashi, P.S., S. Oehen, K. Buerki, H. Pircher, C.T. Ohashi, B. Odernott, B. Malissen, R.M. Zinkernagel, and H. Hengartner. 1991. Ablation of “tolerance” and induction of diabetes by virus infection in viral antigen transgenic mice. Cell. 65:305–317.

25. Zhang, W., J. Sloan-Lancaster, J. Kitchen, R.P. Trible, and L.E. Samelson. 1998. LAT: the ZAP-70 tyrosine kinase subunit to the CD4 family of proteins. J. Exp. Med. 181:1643–1651.

26. June, C.H., and P.S. Rabinovich. 1991. Measurement of intracellular ions by flow cytometry. In Current Protocols in Immunology, Coligan, J.E., A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober, editors. Wiley-InterScience, New York. 5.5.1–5.5.15.

27. Williams, B.L., K.L. Schreiber, W. Zhang, R.L. Wange, L.E. Samekon, P.J. Leibson, and R.T. Abraham. 1998. Genetic evidence for differential coupling of Syk family kinases to the T-cell receptor: reconstitution studies in a ZAP-70-deficient Jurkat T-cell line. Mol. Cell. Biol. 18:1388–1399.

28. Fowlkes, B.J., and D.M. Pardoll. 1989. Molecular and cellular events of T cell development. Adv. Immunol. 44:207–264.

29. Guidos, C.J., J.S. Danska, C.G. Fathman, and L.I. Weissman. 1991. Developmental regulation of lck gene expression: a threshold model. J. Exp. Med. 172:835–845.

30. Lundberg, K., W. Heath, F. Kontgen, F.R. Carbone, and K. Shortman. 1995. Intermediate steps in positive selection: differentiation of CD4+ CD8+ TCR alpha T cells into CD4+ CD8+ TCR alpha T cells. J. Exp. Med. 181:1643–1651.

31. Suzuki, H., J.A. Punt, L.G. Granger, and A. Singer. 1995. Asymmetric signaling requirements for thymocyte commitment to the CD4+ versus CD8+ T cell lineages: a new perspective on thymic commitment and selection. Immunity. 2:413–425.

32. Alberola-Ila, J., K.A. Forbush, R. Seger, E.G. Krebs, and R.M. Perlmutter. 1995. Selective requirement for MAP kinase activation in thymocyte differentiation. Nature. 373: 620–623.

33. Gao, E.K., D. Lo, R. Cheney, O. Kanagawa, and J. Sprent. 1988. Abnormal differentiation of thymocytes in mice treated with cyclosporin A. Nature. 336:176–179.

34. Pages, G., S. Guerin, D. Grall, F. Bonino, A. Smith, F. Anjuere, P. Auburger, and J. Pouyssegur. 1999. Defective thymocyte maturation in p44 MAP kinase (Erk 1) knockout mice. Science. 286:1374–1377.

35. Payne, D.M., A.J. Rossmoando, P. Martino, A.K. Erickson, J.H. Her, J. Shabanowitz, D.F. Hunt, M.J. Weber, and T.W. Sturgill. 1991. Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). EMBO J. 10:885–892.

36. Swat, W., M. Dessing, H. von Boehmcr, and P. Kisielow. 1993. CD69 expression during selection and maturation of CD4+8+ thymocytes. Eur. J. Immunol. 23:739–746.

37. Sant’Angelo, D.B., B. Lucas, P.G. Waterbury, B. Cohen, T. Brabb, J. Goverman, R.N. Germain, and C.A.J. Janeway. 1998. A molecular map of T cell development. Immunity. 9:179–186.

38. Bhandoola, A., R. Cibotti, J.A. Punt, L. Granger, A.J. Adams, S.O. Sharrow, and A. Singer. 1999. Positive selection as a developmental progression initiated by alpha beta TCR signals that fix TCR specificity prior to lineage commitment. Immunity. 10:301–311.

39. Borgulya, P., H. Kishi, Y. Uematsu, and H. von Boehmcr. 1992. Exclusion and inclusion of alpha and beta T cell receptor alleles. Cell. 69:529–537.

40. Monroe, R.J., K.J. Seidl, F. Gaertner, S. Han, F. Chen, J. Sekiguchi, J. Wang, R. Ferrini, L. Davidson, G. Kelsoe, and F.W. Alt. 1999. RAG2:GFP knockin mice reveal novel aspects of RAG2 expression in primary and peripheral lymphoid tissues. Immunity. 11:201–212.

41. Yu, W., Z. Misulovin, H. Suh, R.R. Hardy, M. Jankovic, N. Yannoutsos, and M.C. Nussenweig. 1999. Coordinate regulation of RAG1 and RAG2 by cell type-specific DNA elements 5′ of RAG2. Science. 283:1080–1084.

42. Gong, Q., X. Jin, A.M. Akk, N. Fogel, M. White, G. Gong, J.B. Wardenburg, and A.C. Chan. 2001. Requirement for tyrosine residues 315 and 319 within zeta chain-associated protein 70 for T cell development. J. Exp. Med. 194:507–518.

43. Wildin, R.S., A.M. Garvin, S. Pawar, D.B. Lewis, K.M. Abraham, K.A. Forbush, S.F. Ziegler, J.M. Allen, and R.M. Perlmutter. 1991. Developmental regulation of Ick gene expression in T lymphocytes. J. Exp. Med. 173:383–393.

44. Huesmann, M., B. Scott, P. Kisielow, and H. von Boehmcr. 1991. Kinetics and efficacy of positive selection in the thymus of normal and T cell receptor transgenic mice. Cell. 66: 533–540.

45. Groves, T., M. Parsons, N.G. Miyamoto, and C.J. Guidos. 1997. TCR engagement of CD4+CD8+ thymocytes in vitro induces early aspects of positive selection, but not apoptosis. J. Immunol. 158:65–75.

46. Wilkinson, B., and J. Kaye. 2001. Requirement for sustained MAPK signaling in both CD4 and CD8 lineage commitment: a threshold model. Cell. Immunol. 211:86–95.

47. Vacchio, M.S., and J.D. Ashwell. 1997. Thymus-derived autoreactive T cells in CD8+ TCR alpha T cells. J. Exp. Med. 185:2033–2038.

48. Lucas, B., and R.N. Germain. 1996. Unexpectedly complex regulation of CD4/CD8 coreceptor expression supports a revised model for CD4+CD8+ thymocyte differentiation. Immunity. 5:461–477.

49. Punt, J.A., B.A. Osborne, Y. Takahama, S.O. Sharrow, and A. Singer. 1994. Negative selection of CD4+CD8+ thymocytes by T cell receptor-induced apoptosis requires a costimulatory signal that can be provided by CD28. J. Exp. Med. 179:709–713.
50. Fischer, K.D., A. Zmuldzinas, S. Gardner, M. Barbacid, A. Bernstein, and C. Guidos. 1995. Defective T-cell receptor signalling and positive selection of Vav-deficient CD4^-CD8^- thymocytes. Nature. 374:474–477.

51. Hashimoto, K., S.J. Sohn, S.D. Levin, T. Tada, R.M. Pearlmuter, and T. Nakayama. 1996. Requirement for p56lck tyrosine kinase activation in T cell receptor-mediated thymic selection. J. Exp. Med. 184:931–943.

52. Tarakhovsky, A., M. Turner, S. Schaal, P.J. Mee, L.P. Duddy, K. Rajewsky, and V.L. Tybulewicz. 1995. Defective antigen receptor-mediated proliferation of B and T cells in the absence of Vav. Nature. 374:467–470.

53. Turner, M., P.J. Mee, A.E. Walters, M.E. Quinn, A.L. Mellor, R. Zamoiyska, and V.L. Tybulewicz. 1997. A requirement for the Rho-family GTP exchange factor Vav in positive and negative selection of thymocytes. Immunity. 7:451–460.

54. Zhang, R., F.W. Alt, L. Davidson, S.H. Orkin, and W. Swat. 1995. Defective signalling through the T- and B-cell antigen receptors in lymphoid cells lacking the vav proto-oncogene. Nature. 374:470–473.

55. Daniels, M.A., L. Devine, J.D. Miller, J.M. Moser, A.E. Lukacher, J.D. Altman, P. Kavathas, K.A. Hogquist, and S.C. Jameson. 2001. CD8 binding to MHC class I molecules is influenced by T cell maturation and glycosylation. Immunity. 15:1051–1061.

56. Davey, G.M., S.L. Schober, B.T. Endrizzi, A.K. Dutcher, S.C. Jameson, and K.A. Hogquist. 1998. Preselection thymocytes are more sensitive to T cell receptor stimulation than mature T cells. J. Exp. Med. 188:1867–1874.

57. Lucas, B., I. Stefanova, K. Yasutomo, N. Dautigny, and R.N. Germain. 1999. Divergent changes in the sensitivity of maturing T cells to structurally related ligands underlies formation of a useful T cell repertoire. Immunity. 10:367–376.

58. Dyall, R., and J. Nikolic-Zugic. 1999. The final maturation of at least some single-positive CD4(+)CD8(-) thymocytes does not require T cell receptor-major histocompatibility complex contact. J. Exp. Med. 190:757–764.

59. Akashi, K., M. Kondo, U. von Freeden-Jeffry, R. Murray, and I.L. Weissman. 1997. Bcl-2 rescues T lymphopoiesis in interleukin-7 receptor-deficient mice. Cell. 89:1033–1041.

60. Brugnera, E., A. Bhandoalli, R. Cibotti, Q. Yu, T.I. Guinter, Y. Yamashita, S.O. Sharrow, and A. Singer. 2000. Coreceptor reversal in the thymus: signaled CD4^+CD8^- thymocytes initially terminate CD8^-transcription even when differentiating into CD8^- T cells. Immunity. 13:59–71.

61. Bosselut, R., L. Feigenbaum, S.O. Sharrow, and A. Singer. 2001. Strength of signaling by CD4 and CD8 coreceptor tails determines the number but not the lineage direction of positively selected thymocytes. Immunity. 14:483–494.

62. Yasutomo, K., C. Doyle, L. Miele, C. Fuchs, and R.N. Germain. 2000. The duration of antigen receptor signalling determines CD4^- versus CD8^- T-cell lineage fate. Nature. 404:506–510.

63. Guo, J., A. Hsewari, H. Li, Z. Sun, S.K. Mahanta, D.R. Littman, M.S. Krangel, and Y.W. He. 2002. Regulation of the TCRalpha repertoire by the survival window of CD4^+CD8^- thymocytes. Nat. Immunol. 3:469–476.

64. Petrov, H.T., F. Livak, D.G. Schatz, A. Strasser, L.N. Crispe, and K. Shortman. 1993. Multiple rearrangements in T cell receptor alpha chain genes maximize the production of useful thymocytes. J. Exp. Med. 178:615–622.

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

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