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Analyzing Expression of Perforin, Runx3, and Thpok Genes during Positive Selection Reveals Activation of CD8-Differentiation Programs by MHC II-Signaled Thymocytes

Xiaolong Liu,†‡ Barbara J. Taylor,† Guangping Sun,∗ and Rémy Bosselut†∗

Intrathymic positive selection matches CD4-CD8 lineage differentiation to MHC specificity. However, it is unclear whether MHC signals induce lineage choice or simply select thymocytes of the appropriate lineage. To investigate this issue, we assessed thymocytes undergoing positive selection for expression of the CD8 lineage markers perforin and Runx3. Using both population-based and single-cell RT-PCR analyses, we found large subsets of MHC class II (MHC-II)-signaled thymocytes expressing these genes within the CD4+8int and CD4+8+ populations, but not the CD4+8+ populations of signaling competent mice. This indicates that MHC-II signals normally fail to impose CD4 differentiation and further implies that the number of mature CD8 single-positive (SP) thymocytes greatly underestimates CD8 lineage choice. We next examined whether MHC-II-restricted CD4+8+ thymocytes remain competent to initiate CD8 lineage gene expression. In mice in which expression of the tyrosine kinase Zap70 and thereby TCR signaling were impaired selectively in SP thymocytes, MHC-II-signaled CD4+8+ thymocytes expressed perforin and Runx3 and failed to up-regulate the CD4 marker Thpok. This indicated that impairing TCR signals at the CD4 SP stage switched gene expression patterns from CD4- to CD8-lineage specific. We conclude from these findings that MHC-II-signaled thymocytes remain competent to initiate CD8-specific gene expression even after CD8 down-regulation and that CD4 lineage differentiation is not fixed before the CD4 SP stage. The Journal of Immunology, 2005, 175: 4465–4474.

The choice of CD4 or CD8 lineage during thymocyte differentiation is a central event of T cell development, because it determines the functional fate of developing T cells and is required for proper functioning of the immune system (1–3). Lineage differentiation occurs during the step of T cell development known as positive selection, after thymocytes have commenced CD4 and CD8 expression, rearranged the genes encoding TCR α- and β-chains, and initiated surface TCRαβ expression. During this step, TCR engagement by intrathymic MHC-peptide complexes of appropriate avidity causes such CD4+8+ (double-positive (DP)) TCRαβ+ thymocytes to differentiate into either CD4 single-positive (SP) cells if MHC class II (MHC-II) restricted or CD8 SP cells if MHC-I restricted.

Substantial progress has been made within the past few years regarding important aspects of the lineage choice process, including the intracellular effectors of lineage differentiation (4–11). It remains unclear, however how the concordance of lineage with MHC specificity is established during positive selection. It is currently thought that quantitative attributes of positively selecting TCR signals, including their strength (4, 12–15) or their duration (16–18), direct lineage choice to the appropriate specificity, so that MHC-II-induced TCR signals would be stronger or longer than their MHC-I counterparts and would promote CD4 over CD8 differentiation. Challenges this view, other observations suggest that MHC lineage matching is imperfect (19–24) and that mismatched cells that arise are in most cases eliminated during a subsequent “proofreading” step requiring MHC coengagement of TCR and the matched coreceptor. Another disputed issue is when lineage choice is fixed during the DP to SP transition; although it has been proposed that lineage decisions are established early during positive selection, other experiments suggest that commitment to the CD4 lineage is not fixed until thymocytes have terminated CD8 gene expression (16, 17).

The analysis of these issues is complicated by the lack of early markers of CD4 and CD8 lineage differentiation in the thymus. Indeed, although the mutually exclusive expression of CD4 or CD8 coreceptors defines each lineage in mature T cell populations, changes in CD4 or CD8 expression during positive selection can be transient and are not necessarily indicative of lineage choice (25–28). In particular, a large number of CD8-bound, MHC-I-restricted thymocytes go through a transient CD4+8int phenotypic stage, making CD8 down-regulation useless as an indicator of CD4 differentiation. Reciprocally, the shut-down of CD4 expression, which requires accumulation of Runx3 proteins to activate the CD4 silencer, is a late event during CD8 lineage differentiation (6, 29–32). Thus, the only ex vivo readout of lineage differentiation currently available is the size of CD4 and CD8 SP thymocyte populations, which is affected by the survival of differentiating thymocytes and is therefore a poor indicator of lineage choice in situations in which TCR signaling is compromised (18).

The objective of the present study was to bypass these obstacles to gain insight into how and when lineage differentiation is
matched to MHC specificity. Our approach was to assess MHC-II-restricted thymocytes for the expression of perforin and Runx3, two genes whose expression during positive selection is CD8 lineage specific (18, 31–33); we reasoned that the expression of perforin and Runx3 by MHC-II-signaled cells would be indicative of CD8 lineage, i.e., mismatched, gene expression programs. Two specific questions were investigated using this approach. First, we examined how intrathymic TCR signaling matches lineage to MHC specificity under normal signaling circumstances by assessing the expression of CD8 lineage genes in DP thymocytes intrathymically signaled by MHC-II molecules. Surprisingly, we found that a large fraction of such cells express CD8 lineage markers even though they fail to develop into CD8 SP cells, indicating that the size of CD4 and CD8 SP populations is not representative of lineage differentiation in the thymus. Second, we assessed lineage differentiation in mice genetically modified to impair TCR signaling when MHC-II-signaled thymocytes terminate CD8 expression and become CD4+CD8+. When MHC-II-restricted thymocytes make a mismatched CD8 lineage choice during positive selection, the possibility that lineage choice by MHC-II-restricted thymocytes remains open until late during positive selection.

Materials and Methods

Animals

Mice carrying a Zap70 transgene under control of the human adenosine deaminase promoter (ADA) enhancer and enhancer as generated as previously described (34). Mice used in the present study were derived from two distinct founders (C3 and D5). Wild-type mice (C57BL/6J) were from the National Cancer Institute (NCI) animal facility. B10.BR mice. Mice were analyzed between 5 and 12 wk of age and were deficient for Rag-2 were obtained from Dr. A. Singer (NCI, Bethesda, MD). H-2b AND transgenic mice were generated by intercrossing H-2b/b (H2b) AND transgenic mice were generated by intercrossing H-2b/b (H2b) and B10 BR mice (both from The Jackson Laboratory). H-2b/b AND ADA-Zap55 were obtained by breeding AND H-2b+/ADA-Zap55 and B10.BR mice. Mice were analyzed between 5 and 12 wk of age and were heterozygous for the transgene(s) they carry. Animal procedures used in this study were approved by the NCI animal care and use committee.

Antibodies

The following mAbs were obtained from BD Pharmingen or Caltag Laboratories and used for staining: TCRβ (H57-597), TCR Vα11 (RR8-1), H-2Kb/MHC class I (H2F5), and Runx3 (J255) or of prf1 (35), a member of a cytotoxic program that also includes cathepsin G (Ctg) or Sigma-Aldrich (anti-β-actin, AC-15). Immunoprecipitation and immunoblotting experiments used previously described rabbit antiserum against Zap70 (a gift from Dr. L. Samelson, National Cancer Institute) and Thpok/Krox (37).

Cell preparation and staining

Single-cell thymocyte suspensions were prepared and stained as described previously (34). Cell fluorescence was measured, typically on 10^3 cells, on a two-laser FACS Calibur (BD Biosciences) with 4-decade logarithmic amplification and analyzed using FlowJo software. Live cells were identified by forward light scatter and propidium iodide gating. CD8 down-regulation in ADA-Zap55 and Zap70+/− AND TCR CD4 SP populations was quantified by calculating the ratios of the mean CD8 fluorescence intensity in the CD4 SP gate to that in the DP gate (both as indicated in Fig. 4) in the corresponding mouse; values from three distinct experiments with concurrent analyses of both mouse genotypes were averaged. For analyses shown in Fig. 1C and Fig. 2A and E, thymocyte subpopulations were purified using magnetic beads (Miltenyi Biotec) as previously described (18) and were >98% pure. Single-cell sorting was performed on a specially equipped FACSvantage (BD Biosciences). Single-cell and population-based RT-PCR analyses were performed using procedures and primers described previously (11, 18).

Immunoblot analyses of protein expression

For analyses of Zap70 expression by immunoprecipitation and immunoblotting, cells were lysed at 20 × 10^6/ml on ice for 20 min in 1% Triton X-100 buffer (50 mM Tris (pH 7.4), 1% Triton X-100, 150 mM NaCl, 20 μg/ml leupeptin, and 40 μg/ml aprotinin). Lysates were clarified by centrifugation and processed for anti-Zap70 immunoprecipitation for 2 h at 4°C. Immunoprecipitates were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes, immunoblotted with an anti-Zap70 rabbit antisera and revealed by ECL (Amersham Biosciences) following the manufacturer’s recommendations. For the experiments shown in Fig. 3, sorted cells were directly lysed in Laemmli sample loading buffer; lysates were resolved by SDS-PAGE and immunoblotted with the anti-Zap70 antiserum as described above. Thpok protein expression was analyzed as previously described (11).

Results

Tracking CD8 lineage gene expression in MHC-II-signaled thymocytes

Despite the correspondence between lineage and MHC specificity in mature T cells, it remains unclear at what frequency MHC-II-restricted thymocytes make a mismatched CD8 lineage choice during positive selection. The first objective of the present study was to address this issue by assessing MHC-II-restricted thymocytes for the expression of CD8 lineage markers distinct from CD8 genes themselves. We first tracked the expression of perforin (prf1), a member of a cytotoxic program that also includes cathepsins C and W (33, 38). Perforin mRNA is up-regulated during the
positive selection of CD8 lineage, but not of CD4 lineage thymocytes before detectable changes in CD4 or CD8 surface expression (18, 33, 38). Furthermore, performin mRNA can be detected in thymocytes at the single-cell level by RT-PCR analysis (18). In the present study we first verified that this assay was both sensitive and specific enough for performin mRNA detection during positive selection. Indeed, it detected performin message in 17 of 18 TCR<sup>high</sup> CD8 SP thymocytes, but in none of 17 TCR<sup>high</sup> CD4 SP thymocytes (Table I). Performin-positive cells were present in the subset of DP thymocytes undergoing selection and in the transitional CD4<sup>+</sup>CD8<sup>+</sup> population (Table I) (18), both of which include CD8 lineage thymocytes (25, 26, 29, 38).

We assessed the frequency of performin mRNA-positive thymocytes in mice carrying the MHC-II-restricted AND TCR transgene. In H-<sup>2</sup><sup>b</sup> (I-A<sup>b</sup>+) AND transgenic mice, large numbers of CD4 SP thymocytes expressing the clonotypic TCR V<sub>α11</sub> chain develop, whereas there is no detectable population of V<sub>α11</sub><sup>high</sup> CD8 SP thymocytes (39) (illustrated in Fig. 1A), suggesting that no CD8 differentiation is taking place. Challenging this conclusion, single-cell analyses showed that 76% of DP and 38% of CD4<sup>+</sup> CD8<sup>+</sup> AND thymocytes expressed performin, suggesting that they were undergoing CD8 lineage differentiation (Table II; see Fig. 1A for a depiction of the sorting gates used in this analysis). In contrast, virtually all (20 of 21, >95%) CD4<sup>+</sup> CD8<sup>+</sup> cells from AND TCR mice were performin negative, similar to mice with a diverse TCR repertoire. In agreement with these results, population-based RT-PCR analyses showed a >30-fold reduction in performin gene expression during the DP to CD4 SP transition (Fig. 1B). Performin expression was not detected in AND DP thymocytes deficient for the tyrosine kinase Zap70, a critical intermediate in TCR signal transduction (35, 40, 41), indicating that it required intrathymic TCR signaling (Fig. 1C) (18). A similar pattern of expression was observed for Runx3 (Fig. 1, B and C), a gene that is expressed in a CD8 lineage-specific manner in the thymus and is required for the proper silencing of CD4 during CD8 T cell differentiation, but that is not

FIGURE 2. Expression and function of the ADA-Zap70 transgene in D5-line mice. A, Expression of endogenous and transgenic Zap70 molecules was analyzed by immunoprecipitation and immunoblotting with a rabbit polyclonal Ab against Zap70 in unseparated thymocytes (lanes 1–4) or in microbead-purified thymocyte or lymph node (LN) T cell populations (lanes 5–8). The reduced mobility of transgenic Zap70 molecules is due to their C-terminal Myc epitope tag. As assessed by densitometric analyses, the expression of transgenic Zap70 (lane 3) was 1.2 times that of endogenous Zap70 (lane 1). Relative to that in DP thymocytes (lane 5), the expression of transgenic Zap70 molecules was 0.37 in CD4 SP (lane 6), 0.2 in CD8 SP (lane 7), and 0.19 in LN T (lane 8) cells. B, Expression of transgenic Zap70 was assessed on thymocytes surface stained with Abs against CD4 and CD8 and intracellularly stained with an anti-Myc tag Ab after fixation and permeabilization. Fluorescence intensities are shown as overlaid histograms drawn on gated populations of ADA-ZapD5 (plain lines) or nontransgenic Zap70<sup>+/+</sup> thymocytes (gray-filled histograms). Similar levels and patterns of expression were observed in Zap70<sup>+/+</sup> mice expressing the transgene. C, Thymocyte single-cell suspensions were prepared, enumerated by trypan blue exclusion (number of live cells given as the mean ± SEM; n, number of mice analyzed), and surface stained with Abs against CD4, CD8, and TCR<sub>B</sub>. Representative contour plots of thymocyte CD4 and CD8 expression are shown (left) and were used to define CD4 and CD8 SP populations on which surface expression of TCR<sub>B</sub> is shown (single color histograms, right). D, Surface expression of CD69 on gated CD4<sup>+</sup> and TCR<sub>B</sub><sup>high</sup>CD4<sup>+</sup>CD8<sup>+</sup> thymocytes from D5-line (plain trace) and A-line (dashed trace) ADA-Zap70 transgenic mice. Light gray-filled histograms indicate CD69 expression on the corresponding population from Zap70<sup>+/+</sup> mice, whereas the dark gray histogram (top) shows background staining with an irrelevant Ab. E, Microbead-purified CD4 or CD8 SP thymocytes (center and right) or unseparated thymocytes (left; analysis gated on DP cells) were either stimulated by plate-bound anti-TCR<sub>B</sub> Abs (10 µg/ml, 18 h at 37°C; plain lines) or left untreated (gray-filled histograms). Cells were then analyzed by three-color flow cytometry. Single-color histograms show CD25 expression on CD4 or CD8 SP cells (center and right). Anti-TCR<sub>B</sub> stimulation normally up-regulated CD69 on ADA-ZapD5 DP thymocytes (left).
AND TCR thymocytes, I-A<sup>b</sup>

**A**

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**B**

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### Table 1. Analyses of perforin mRNA expression by single-cell RT-PCR in mice with endogenous TCR repertoire<sup>a</sup>

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<td>3/15&lt;sup&gt;c&lt;/sup&gt;</td>
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<td></td>
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<td>9/10</td>
<td>8/8</td>
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<tr>
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<td>1/7</td>
<td>2/12</td>
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<sup>a</sup>In each experiment, thymocytes were directly single-cell sorted using a specially equipped FACS Vantage cell sorter. The frequency of perforin mRNA-positive cells within each population is given as the ratio of perforin-positive wells over wells positive for β-actin mRNA (all perforin-positive wells were β-actin-positive). Approximately one-third of wells failed to give rise to any amplified band. When two or more experiments (Expt.) were performed, they were carried on cells collected during separate sorts from at least two distinct mice.

<sup>b</sup>Cells are designated by the gate used for sorting.

<sup>c</sup>Data on CD4<sup>+</sup>8<sup>int</sup> thymocytes are from Ref. 18 and are provided for comparison purposes only.

### FIGURE 3. Zap70 expression and intrathymic TCR signaling in AND TCR transgenic ADA-ZapD5 mice. A, Immunoblot analyses compared the expression of transgenic Zap70 in sorted AND ADA-ZapD5 thymocytes and of endogenous Zap70 in the corresponding populations of AND Zap70<sup>+/+</sup> mice. Sorted cells (10<sup>5</sup> cell equivalents/lane) were analyzed by immunoblotting first for Zap70 and then for β-actin (by stripping and reblotting of the same membrane). Identical results were obtained on H-2<sup>b</sup> and H-2<sup>b</sup> background mice in three such experiments. B, Expression of transgenic Zap70 molecules was assessed in AND ADA-ZapD5 thymocytes by intracellular staining and flow cytometry, as described in Fig. 2B. C, Intrathymic TCR signaling was evaluated by flow cytometric measurement of CD5 or CD69 surface expression in DP, CD4<sup>+</sup>8<sup>int</sup>, and CD4<sup>+</sup>8<sup>-</sup> cells from ADA-ZapD5 and Zap70<sup>+/+</sup> AND mice on three-color-stained thymocytes (CD4, CD8, and either CD5 or CD69). Surface expression of CD5 (top) or CD69 (bottom) of AND ADA-ZapD5 cells (plain line) is overlaid on that on their Zap70<sup>+/+</sup> counterparts (gray-filled histograms).

CD8 lineage gene expression as a tool to track the CD4 lineage commitment checkpoint

The presence of large numbers of MHC-II-restricted thymocytes initiating CD8 differentiation indicated that MHC-II signals fail to impose CD4 choice. The fact that these cells were found in the DP but not in the CD4 SP compartment was consistent with the possibility, suggested by in vitro analyses (17), that lineage choice is decided early in DP thymocytes, so that only CD4 lineage cells reach the CD4 SP stage. Contrasting with this possibility, it has been proposed that CD8 down-regulation is a noncommitting response of thymocytes to TCR signals, and that CD4 choice is decided during the down-regulation of CD8, i.e., beyond the DP stage, by the persistence of TCR signaling despite CD8 down-regulation (16, 28, 43). This perspective (known as kinetic signaling) raises the possibility that thymocytes reach a phenotypic CD4 part of the cytotoxic program (6). We conclude from these analyses that a large fraction of MHC-II-signal thymocytes from AND mice activate gene expression programs characteristic of CD8 lineage differentiation.
Disrupting TCR signaling in SP thymocytes

Our approach to impair TCR signaling in CD4 SP thymocytes was to reduce the expression of the tyrosine kinase Zap70 in these cells. We used a previously described transgenic cassette based on a human ADA gene enhancer turned off during the DP to SP transition (44). In the line used in the present study (referred to as D5), transgenic Zap70 expression in DP thymocytes exceeded that of endogenous Zap70 and was reduced by 65–80% in CD4 and CD8 SP thymocytes and peripheral T cells (Fig. 2A, lanes 1, 3, and 5–8). Intracellular staining and flow cytometry detected transgene expression in all DP thymocytes and in CD4+8int thymocytes (Fig. 2B). Another line (C3) had an identical pattern and levels of Zap70 expression and gave rise to identical phenotypes after backcrossing to the Zap70+/- background (data not shown). The overall expression of the transgene in the D5 line was ~4 times greater than that in the previously described A-line injected with the same construct (34) (Fig. 2A, lanes 2 and 3). Importantly however, the developmental pattern of expression of the transgene was the same in both lines (Fig. 2, A and B) (34).

Unlike Zap70+/- mice (35, 41), Zap70-/- mice expressing the ADA-Zap70 (D5) transgene (hereafter referred to as D5-line or ADA-ZapD5 mice) had SP thymocytes and mature T cells (Fig. 2C) and data not shown). The numbers of both CD4 SP thymocytes and CD4 peripheral T cells were normal. In contrast, there was a 2-fold increase in the number of CD8 SP thymocytes, contrasting with a 50% reduction in the numbers of spleen CD8 T cells, a phenotype linked to impaired terminal maturation of CD8 SP thymocytes (X. Liu et al., manuscript in preparation). We took two approaches to analyze TCR signal transduction in D5-line thymocytes. For DP and CD4+8int thymocytes, we assessed ex vivo expression of surface CD69, a marker of intrathymic TCR signaling in these cells (45). CD69 levels in D5-line DP or CD4+8int thymocytes were identical to those in the corresponding Zap70+/- subset, consistent with the pattern of Zap70 expression (Fig. 2D). This contrasted with A-line mice (Fig. 2D), in which CD69 expression was normal in DP thymocytes but impaired in CD4+8int cells because of insufficient transgenic Zap70 amounts from that stage on (34). Ex vivo CD69 levels are not indicative of TCR signal transduction in SP thymocytes, because CD69 is normally down-regulated in these cells. Consequently, we assessed Zap70 function in SP thymocytes by measuring CD25 up-regulation after in vitro TCR stimulation (46). CD25 up-regulation was strongly impaired in D5-line CD4 and CD8 SP cells (Fig. 2E), demonstrating reduced TCR signal transduction in these cells (note that TCR stimulation fails to up-regulate CD25 even in Zap70+/- DP thymocytes). We conclude from these analyses that transgenic Zap70 in D5-line mice promotes TCR signaling in DP and CD4+8int, but not in SP, thymocytes; in contrast, transgenic Zap70 in A-line mice promotes TCR signaling in DP thymocytes only (34). As a result, D5-line mice generate CD4 SP thymocytes that are signaling deficient, whereas A-line mice generate no CD4 SP thymocytes (34). Thus, D5-line, but not A-line, mice were used to examine whether the persistence of TCR signaling in CD4 SP thymocytes affects lineage differentiation.

CD8 lineage choice remains open throughout CD8 down-regulation

To examine whether CD8 lineage choice remained open after CD8 down-regulation, we generated ADA-ZapD5 mice transgenic for the AND TCR. The expression of the AND TCR transgene did not affect the pattern of transgenic Zap70 expression (Fig. 3A); compared with endogenous Zap70 in AND Zap70+/- mice, transgenic Zap70 expression in AND ADA-ZapD5 mice was slightly higher in DP thymocytes, identical in the CD4+8int population, and markedly lower in CD4 SP cells. Intracellular staining and flow cytometry confirmed that the transgene was expressed in all DP and CD4+8int thymocytes and was down-regulated in CD4 SP cells (Fig. 3B). Intrathymic TCR signaling (Fig. 3C), analyzed by measuring the expression of the sensitive indicator CD5 (47), was as predicted by this expression pattern. CD5 expression in AND ADA-ZapD5 DP or CD4+8int populations was unmodal and superimposable to that of their Zap70+/- counterparts (and the same was true of CD69); in contrast, CD5 expression was slightly lower in ADA-ZapD5 than in Zap70+/- CD4+8int thymocytes.

Analyses of positive selection on the H-2b background revealed two salient differences between ADA-ZapD5 and Zap70+/- AND thymi. First, the number of CD4 SP thymocytes in ADA-ZapD5 mice was reduced by 55% (Fig. 4, left, and bar graph). CD4 SP thymocytes did not undergo negative selection from A-line mice (Fig. 2E) or in ADA-ZapD5 mice (Fig. 4, right), in contrast to A-line mice, which generate no CD4 SP thymocytes (34). Thus, unlike A-line mice, D5-line mice provide a relevant model of SP lineage choice.

Table II. Single-cell RT-PCR analyses of perforin mRNA expression in AND TCR transgenic mice

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<td></td>
<td>CD4+84</td>
<td>9/11</td>
<td>4/5</td>
<td>nd</td>
<td>81</td>
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</table>

a Experiments were performed and results are displayed as described in the legend to Table I. Frequencies were calculated by averaging data from the two or three experiments (Expt.) performed. nd. Not done.
AND TCR thymocytes, I-A\textsuperscript{b}

<table>
<thead>
<tr>
<th>ADA-Zap70</th>
<th>Zap70</th>
<th>All</th>
<th>CD4\textsuperscript{+8\textsuperscript{−}}</th>
<th>CD4\textsuperscript{+8\textsuperscript{+}}</th>
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<tr>
<td>+/+</td>
<td>210 ± 23 (n = 5)</td>
<td># x 10\textsuperscript{4}</td>
<td>08</td>
<td>59</td>
</tr>
<tr>
<td>−/−</td>
<td>191 ± 22 (n = 5)</td>
<td>99</td>
<td>85</td>
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</tr>
<tr>
<td>−/−</td>
<td>37 ± 3 (n = 3)</td>
<td>N/A</td>
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Vo\textalpha\textsuperscript{11}(n = 10\textsuperscript{6})

<table>
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<tr>
<th>CD4\textsuperscript{+}/CD8\textsuperscript{−}</th>
<th>CD4\textsuperscript{+}/CD8\textsuperscript{+}</th>
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<td>0</td>
<td>0</td>
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<tr>
<td>4</td>
<td>12</td>
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FIGURE 4. T cell differentiation in H-2\textsuperscript{b} Zap70\textsuperscript{+/−} and ADA-ZapD5 AND TCR transgenic mice. Thymocyte single-cell suspensions were prepared as described in Fig. 2C from H-2\textsuperscript{b} mice expressing the AND TCR transgene and surface stained with Abs against CD4, CD8, and the transgenic Vo\textalpha\textsuperscript{11} TCR α-chain. Shown are representative contour plots of thymocyte CD4 and CD8 expression and single-color histograms of Vo\textalpha\textsuperscript{11} expression on CD4\textsuperscript{+}8\textsuperscript{−} and CD4\textsuperscript{+}8\textsuperscript{+} thymocyte populations (gated as indicated on contour plots). Bar graphs indicate the numbers (average ± SEM) of Vo\textalpha\textsuperscript{11}\textsuperscript{high} CD4\textsuperscript{+}8\textsuperscript{−} or CD4\textsuperscript{−}8\textsuperscript{−}CD8\textsuperscript{+} thymocytes. Note that different scales were used on the left and right sides to increase the resolution of the bar graph. T cell development is arrested at a CD8\textsuperscript{−}CD69\textsuperscript{−}CD4\textsuperscript{+}8\textsuperscript{+} stage in AND transgenic Zap70\textsuperscript{+/−} mice (18).

To further examine lineage choice by AND ADA-ZapD5 thymocytes, we examined their expression of perforin and Runx3. Consistent with the expression pattern of the ADA-Zap70 transgene, there was no difference between Zap70\textsuperscript{+/−} AND and ADA-ZapD5 AND mice for the expression of both markers in DP and CD4\textsuperscript{+}8\textsuperscript{+} populations (Fig. 5, left two columns). In contrast, both perforin and Runx3 were expressed at higher levels in ADA-ZapD5 than in Zap70\textsuperscript{+/−} CD4 SP populations, suggesting that a fraction of the ADA-ZapD5 CD4 SP thymocytes was initiating CD8 differentiation. Single-cell analysis of perforin expression also supported this idea (Table II). Although the frequencies of perforin-positive cells in the CD4\textsuperscript{+}8\textsuperscript{−} and DP populations were similar in ADA-ZapD5 and Zap70\textsuperscript{+/−} AND mice, the frequency of perforin-positive cells within the CD4\textsuperscript{+}8\textsuperscript{−} population was almost 10-fold greater in ADA-ZapD5 (nine of 21, 43%) than in Zap70\textsuperscript{+/−} mice (one of 21, 4.8%). Taking into account the respective sizes of CD4\textsuperscript{+}8\textsuperscript{−} populations in both lines, this translated into a 4-fold increase in the absolute numbers of CD4\textsuperscript{+}8\textsuperscript{−} perforin-positive cells (16 × 10\textsuperscript{6} vs. 3.9 × 10\textsuperscript{6}, respectively; absolute numbers calculated from the data shown in Fig. 4 and Table II), also supporting the concept that a large number of ADA-ZapD5 AND CD4 SP thymocytes had initiated CD8 lineage gene expression. Of note, most of the ADA-ZapD5 AND CD4\textsuperscript{+}8\textsuperscript{+} cells expressed perforin, confirming that they belonged to the CD8 lineage. We conclude from these analyses that almost half of CD4 SP thymocytes in ADA-ZapD5 AND had activated CD8 lineage gene expression programs.

If the expression of CD8 lineage markers by a large subset of the ADA-ZapD5 AND CD4 SP population is indicative of CD8 choice, the expression of CD4 lineage markers should be impaired in this population. We and others (10, 11) have recently shown that the transcription factor Thpok (also known as cKrox) is up-regulated during the positive selection of CD4 but not of CD8 T cells and that it promotes CD4 lineage differentiation. Consequently, we assessed Thpok mRNA and protein expression during positive selection in Zap70\textsuperscript{+/−} and ADA-ZapD5 AND mice (Fig. 6A). Thpok mRNA was gradually up-regulated during the DP to CD4 SP transition in AND Zap70\textsuperscript{+/−} mice. In the CD4\textsuperscript{−}8\textsuperscript{+} population, Thpok mRNA expression was lower in ADA-ZapD5 than in Zap70\textsuperscript{+/−} mice, indicating impaired CD4 differentiation. In contrast, Thpok mRNA levels were normal in ADA-ZapD5 AND DP and CD4\textsuperscript{+}8\textsuperscript{+} populations. Similar results were obtained from analyses

FIGURE 5. Impaired TCR signaling in CD4\textsuperscript{−}8\textsuperscript{+} thymocytes up-regulates CD8 lineage-specific gene expression. Thymocyte populations were sorted from mice of the indicated genotype (left columns) and analyzed by RT-PCR for perforin (prf1), Runx3, and β-actin (Actb) mRNA expression as described in Fig. 1B. Wedges indicate RNA input (left to right, 100, 30, 10, and 3 cell equivalents).
H-2bxk mice expressing the AND TCR transgene and surface stained with Abs against CD4, CD8, and the transgenic Zap70 transgenic.

**FIGURE 7.** CD8 SP thymocyte development in H-2^bxk ADA-ZapD5 AND TCR transgenic mice. Thymocytes were prepared as described in Fig. 4 from DP and CD4 TCR signaling in ADA-ZapD5 thymocytes operates normally in down-regulated the CD4-differentiating factor Thpok. Because I-E^k promotes CD8 T cell-positive selection in CD4-deficient AND mice, indicating that I-E^k enables CD4-independent signaling by the AND TCR (13). In this second perspective, selection of ADA-ZapD5 AND thymocytes by I-E^k should result in the generation of CD8 SP thymocyte populations. Analyses of H-2^bxk ADA-ZapD5 AND mice fulfilled the second prediction. The number of CD4 SP thymocytes was reduced in H-2^bxk mice to the same extent as in H-2^b mice (Fig. 7, bar graph); these CD4 SP thymocytes expressed perforin and Runx3 and had reduced Thpok expression compared with their Zap70^+/+ counterparts (data not shown). Unlike on the H-2^b background, however, distinct populations of Vα11^high^ CD8 lineage (CD4^-^8^+^ and CD4^-^8^-^) thymocytes were readily apparent in H-2^bxk ADA-ZapD5 AND mice (Fig. 7) even though they failed to give rise to detectable peripheral CD8 T cell populations (data not shown). These observations support the conclusion that impaired TCR signaling after CD8 down-regulation redirects thymocytes to the CD8 lineage.

**Discussion**

The present study reports two main findings. First, it shows that surprisingly large numbers of MHC-II-signaled thymocytes initiate CD8-specific gene expression programs even in normal signaling circumstances, presumably before being eliminated through programmed cell death. This suggests that the size of CD8 SP populations massively underestimates CD8 lineage choice and that the matching of CD8 lineage to MHC-I specificity in mature T cell populations has an important proofreading component. Second, the present report shows that MHC-II-signaled thymocytes remain competent to initiate CD8 lineage gene expression programs until the cessation of CD8 expression, indicating that lineage-specific gene expression is not fixed until late in CD4 T cell development.

**Unsuspected CD8 differentiation by MHC-II-signaled thymocytes in vivo**

Our analyses show that even under normal signaling circumstances, large numbers of immature MHC-II-restricted thymocytes express the CD8 lineage-specific genes prf1 and Runx3. Although

To support this conclusion, we analyzed positive selection in ADA-ZapD5 AND mice expressing I-E^k, the restricting element for the AND TCR. Because I-E^k engages this TCR with greater avidity than I-A^b and thereby promotes greater signaling (13, 48), two opposite but nonmutually exclusive predictions could be advanced regarding positive selection in these mice. First, it was possible that the greater signals provided by I-E^k would prevent CD8 lineage redirection and restore CD4 differentiation. Alternatively, it was possible that I-E^k signals would fail to prevent CD8 lineage redirection, but would be sufficient to allow the survival of CD8-directed AND thymocytes after they had down-regulated CD4. Indeed, I-E^k promotes CD8 T cell-positive selection in CD4-deficient AND mice, indicating that I-E^k enables CD4-independent signaling by the AND TCR (13). In this second perspective, selection of ADA-ZapD5 AND thymocytes by I-E^k should result in the generation of CD8 SP thymocyte populations. Analyses of H-2^bxk ADA-ZapD5 AND mice fulfilled the second prediction. The number of CD4 SP thymocytes was reduced in H-2^bxk mice to the same extent as in H-2^b mice (Fig. 7, bar graph); these CD4 SP thymocytes expressed perforin and Runx3 and had reduced Thpok expression compared with their Zap70^+/+^ counterparts (data not shown). Unlike on the H-2^b background, however, distinct populations of Vα11^high^ CD8 lineage (CD4^-^8^+^ and CD4^-^8^-^) thymocytes were readily apparent in H-2^bxk ADA-ZapD5 AND mice (Fig. 7) even though they failed to give rise to detectable peripheral CD8 T cell populations (data not shown). These observations support the conclusion that impaired TCR signaling after CD8 down-regulation redirects thymocytes to the CD8 lineage.

**FIGURE 7.** CD8 SP thymocyte development in H-2^bxk ADA-ZapD5 AND TCR transgenic mice. Thymocytes were prepared as described in Fig. 4 from H-2^bxk mice expressing the AND TCR transgene and surface stained with Abs against CD4, CD8, and the transgenic Vα11 TCR α-chain. Shown are representative contour plots of thymocyte CD4 and CD8 expression and single-color histograms of Vα11 expression on thymocyte populations. Bar graphs indicate the average numbers of Vα11^high^ CD4^-^8^+^ or CD4^-^8^-^ thymocytes (n, number of mice analyzed; error bars, SEM). T cell development in AND transgenic Zap70^-/-^ H-2^bxk mice was arrested at the same CD5^low^CD69^-^ CD4^-^8^-^ developmental stage as in their H-2^bxk counterparts (not shown).

**FIGURE 6.** Impaired TCR signaling in CD4^-^8^-^ thymocytes and Thpok expression. Thymocyte populations were sorted from mice of the indicated genotype (left columns) and analyzed for the expression of Thpok and β-actin mRNAs (A) as described in Fig. 1B. Wedges indicate RNA input (left to right, 100, 30, 10, and 3 cell equivalents). B, Sorted thymocytes (10^6/lane) were analyzed for expression of Thpok protein by immunoprecipitation and immunoblotting with an anti-Thpok rabbit antiserum (11, 37).

of Thpok protein expression (Fig. 6B). Given the role of Thpok during CD4 T cell development, these observations indicate that CD4 differentiation is impaired in the CD4 SP population of ADA-ZapD5 AND mice.

In summary, gene expression analyses indicate that a large subset of the CD4 SP population from ADA-ZapD5 AND mice have up-regulated the CD8 lineage markers perforin and Runx3 and down-regulated the CD4-differentiating factor Thpok. Because TCR signaling in ADA-ZapD5 thymocytes operates normally in DP and CD4^-^8^-^ but not in CD4 SP thymocytes, these observations support the possibility that the ability of MHC-II-restricted thymocytes to abort CD4 differentiation and to initiate CD8 differentiation in response to reduced TCR signaling persists until the completion of CD8 down-regulation.
it could be argued that perforin expression reveals the increased representation of cytotoxic CD4 T cell populations that are normally of low frequency (such as NK T cells), this is not the case for Runx3, which is not involved in cytotoxic differentiation (6). Rather, Runx3 is required for normal CD4 silencing during positive selection and is therefore an essential component of the CD8 lineage differentiation program in the thymus (6, 31). Thus, we interpret our findings to mean that a substantial fraction of MHC-II-signalized thymocytes initiates a CD8 differentiation program. Although our in vivo approach could not address whether CD4^8^- perforin-expressing thymocytes are fully competent to differentiate into mature CD8 T cells, this possibility is supported by previous reports demonstrating that intrathymically signalized thymocytes that express CD4 but not CD8 retain the ability to differentiate into CD8 SP cells in vitro or after adoptive transfer (16, 28, 49).

Our observations imply that CD4-CD8 lineage choice cannot be analyzed by simply comparing the sizes of CD4 and CD8 SP thymocyte populations. The absence of CD8 SP thymocytes does not equate with the absence of CD8 lineage differentiation, whereas a substantial fraction of CD8 lineage cells may appear as CD4^8^- in vivo in circumstances of altered cell signaling or gene expression. Although the frequency of MHC-II-signalized thymocytes with CD8 lineage gene expression within CD4-positive populations (DP and CD4^8^int) was higher in AND TCR transgenic mice than in mice with a diverse TCR repertoire (MHC-I/H11002), the presence of such cells was not unique to TCR transgenic models. We favor the possibility that the higher frequency of cells with CD8 lineage gene expression in AND thymi results from the inability of TCR transgenic thymocytes to sustain TCR signaling because of limited ligand availability, a feature common to the AND and other TCR transgenic models (50, 51); however, it cannot be excluded that this higher frequency reflects some unique property of the AND transgene.

**Lineage differentiation and TCR signaling**

In a previous study we found that the duration of intrathymic TCR signaling affected lineage differentiation, with long signals promoting CD4 differentiation and short signals promoting CD8 differentiation (18). Specifically, our previous report showed that cessation of TCR signaling in CD4^8^int populations redirected MHC-II-signalized cells toward the CD8 lineage. The present study adds two elements to these previous findings. First, it extends the downstream boundary of the developmental window during which thymocytes remain competent to initiate CD8 differentiation. Indeed, cessation of transgenic Zap70 expression in the previously reported A-line mice (18) resulted in a CD4^8^int arrest and the absence of an intrathymic CD4^8^- population. In contrast, TCR signaling in the D5 line used in the present study is preserved in CD4^8^int thymocytes and is not impaired before the SP stage. As a result, D5-line mice have CD4^8^- thymocytes that are incompetent for TCR signaling, allowing us to demonstrate that lineage differentiation is still affected by TCR signaling at this late developmental stage. It is notable that reduced Zap70 expression in CD4^8^- cells impaired but did not preclude CD4 differentiation, presumably because their residual Zap70 allows some cells to sustain signaling until the CD4 commitment checkpoint, whereas most other cells fail to do so and initiate CD8 lineage gene expression.

The second important difference from the previously reported A line (18) regards the level of Zap70 expression in DP thymocytes. Because Zap70 levels were lower in A-line than in Zap70^+/+ DP thymocytes, it has been argued that CD4 differentiation in A-line mice was impaired because TCR signaling was insufficient, rather than because it was transient (52). This objection does not apply to the D5 line, in which CD4 differentiation is impaired despite sufficient Zap70 expression in DP and CD4^8^int thymocytes. Thus, the present study supports the hypothesis that persistence of TCR signaling throughout positive selection, rather than its initial intensity in DP cells, is important for CD4 differentiation (16, 43).

**CD4 commitment as a late event during positive selection**

The fact that CD4^8^- thymocytes can initiate CD8 lineage gene expression indicates that CD4 lineage commitment (understood as the loss of CD8 potential) is a late event during CD4 T cell differentiation. Previous attempts at mapping a putative CD4 commitment checkpoint in the thymus reached conflicting conclusions. In vitro experiments using a two-step culture system found that CD4 or CD8 lineage commitment precedes any change in CD4 or CD8 gene expression, supporting the simple view that lineage commitment occurs at the DP stage (17). In contrast, other studies showed that CD4^8^- populations that have ceased CD8 gene expression retain CD8 precursor activity in cell culture or intrathymic adoptive transfer experiments (16, 28), suggesting that CD8 down-regulation is a noncommitting response to intrathymic TCR engagement. Using a genetic strategy that bypasses the limitations inherent to these indirect approaches, our present findings strongly support this second view, because 1) CD8 lineage gene expression could be initiated in CD4^8^- thymocytes and 2) conditions that promoted CD8 lineage gene expression in the CD4^8^- population also impaired the expression of Thpok, a factor essential to CD4 differentiation (10). Together, these findings support the conclusion that a subset of CD4 SP thymocytes can lose their potential for CD4 differentiation and attempt CD8 differentiation, implying that they remain CD4 uncommitted.

CD4 SP thymocytes are known to comprise at least two subpopulations. A semi mature population, characterized by high level CD24 and CD69 expression, is thought to remain sensitive to negative selection signals as it responds to high avidity TCR engagement by undergoing programmed cell death (53). A more mature population is composed of CD24^low/CD69^low cells that respond to high avidity TCR engagement by proliferation and effector differentiation (54). Although the latter population itself appears to be heterogeneous (55), it is believed to include cells nearing the completion of their development and candidates for thymus exit (56). Thus, it is tempting to propose that the loss of CD8 potential, i.e., CD4 lineage commitment, occurs during these late steps of CD4 cell maturation even though we found that impaired Zap70 expression in CD4 SP cells did not prevent CD24 down-regulation.

**CD8 lineage choice as an escape pathway during the positive selection of MHC-II-restricted thymocytes**

We previously proposed that the concordance between CD8 lineage and MHC-I specificity in the mature T cell repertoire is established by a two-step intrathymic mechanism (18). The first step would prevent the CD4 differentiation of MHC-I-restricted thymocytes and operate until intrathymically TCR-signalized thymocytes have down-regulated CD8 gene expression; at this stage, the persistence of TCR signaling despite CD8 down-regulation would be required for CD4 differentiation (the kinetic signaling hypothesis (16, 43)). Thymocytes able to sustain TCR signaling despite CD8 down-regulation are mostly MHC-II-restricted cells, because MHC-I-induced intrathymic TCR signaling is CD8 dependent (57). Thymocytes unable to sustain TCR signaling during this first step of positive selection would adopt a CD8 fate, thereby confining CD4 differentiation to MHC-II-signalized thymocytes. A second TCR- and coreceptor-signalized developmental step was then proposed to proofread CD8 choice and eliminate mismatched CD8
lineage MHC-II-restricted cells. This proofreading step was previously shown (18) to target thymocyte survival, possibly through TCR-mediated IL-7R up-regulation (16, 58). Other findings suggest that it affects additional aspects of the DP to CD8 SP transition, such as thymocyte maturation and export (X. Liu et al., manuscript in preparation) or CD4 silencing (18, 58). In this two-step perspective, mismatched CD8 choice by MHC-II-signalized thymocytes would be a nonreversible event, because Runx3 up-regulation would direct CD4 shut-down and thereby additional reduction of MHC-II-induced signaling. Thus, although our present approach did not directly address this issue, we favor the possibility that the initiation of CD8 lineage gene expression commits thymocytes to a CD8 fate.

The present study adds two elements in support of this two-step hypothesis. First, unlike in previous reports (25, 29), we directly show that large numbers of MHC-II-restricted thymocytes (perhaps those with limiting avidity for or access to MHC peptide ligands) initiate CD8 lineage gene expression even under normal signaling circumstances. Secondly, our findings support the idea that mismatched CD8 lineage MHC-II-restricted thymocytes are unavoidable byproducts of a stringent mechanism preventing CD4 choice by MHC-I-signalized cells, rather than arising stochastically (19, 21, 24, 59–61).

The fact that impaired TCR signaling in CD4+8–low cells did not affect the size of CD4 SP thymocyte populations in mice expressing endogenously rearranged TCRs does not restrict the validity of our conclusions to AND TCR mice. It is likely that the normal size of the CD4 SP population in non-TCR transgenic D5 lineage thymus is due to compensatory mechanisms that operate in the presence of a highly diverse TCR repertoire, but not when TCR specificity is fixed. One simple possibility is that Zap70 down-regulation in CD4 SP thymocytes impairs the deletion of cells that carry high avidity TCRs and normally undergo negative selection at this stage (53). Furthermore, the expression of selecting ligands is limiting in TCR transgenic mice (50, 51), and it is possible that this exacerbates the developmental consequences of Zap70 down-regulation in the AND mice used in the present study. Conversely, impaired TCR signaling in CD4 SP thymocytes of mice expressing a diverse TCR repertoire resulted in enlarged CD8 SP populations. Although the terminal maturation of MHC-I-restricted CD8 SP thymocytes is also affected by impaired signaling (X. Liu et al., manuscript in preparation), our findings in H-2b/H11002 mice suggest that this excess CD8 SP population is in part MHC-II restricted.

The ability to detect CD8 lineage choice before the CD8 SP stage allowed us to reinterpret the role of antiapoptotic molecules, such as Bcl-2, in CD8 lineage differentiation. Transgenic expression of Bcl-2 results in the generation of MHC-II-restricted CD8 SP thymocytes (e.g., in AND TCR transgenic or MHC-I−/− mice), but not of MHC-I-restricted CD4 SP thymocytes (62, 63), raising the possibility that Bcl-2 promotes, directly or indirectly, CD8 lineage differentiation. For instance, it had been found that Bcl-2 overexpression interferes with T cell signaling (64), suggesting that transgenic Bcl-2 might promote CD8 choice by reducing TCR signals. In fact, our results demonstrate that large numbers of MHC-II-restricted thymocytes initiate CD8 differentiation even in the absence of the bcl-2 transgene, supporting the view that the bcl-2 transgene does not act to promote CD8 lineage differentiation, but simply reveals CD8 lineage thymocytes that would otherwise die before reaching the CD8 SP stage.

Acknowledgments

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Disclosures

The authors have no financial conflict of interest.

References

LINEAGE DIFFERENTIATION OF MHC-II SIGNALED THYMOCYTES