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Positive Selection as a Developmental Progression Initiated by αβTCR Signals that Fix TCR Specificity prior to Lineage Commitment

Avinash Bhandoola, Ricardo Cibotti, Jennifer A. Punt,† Larry Granger, Anthony J. Adams, Susan O. Sharrow, and Alfred Singer*
Experimental Immunology Branch
National Cancer Institute
National Institutes of Health
Bethesda, Maryland 20892

Summary

During positive selection, immature thymocytes commit to either the $CD4^+$ or $CD8^+$ T cell lineage ("commitment") and convert from short-lived thymocytes into long-lived T cells ("rescue"). By formal precursor-progeny analysis, we now identify what is likely to be the initial positive selection step signaled by αβTCR, which we have termed "induction". During induction, RAG mRNA expression is downregulated, but lineage commitment does not occur. Rather, lineage commitment (which depends upon the MHC class specificity of the αβTCR) only occurs after downregulation of RAG expression and the consequent fixation of αβTCR specificity. We propose that positive selection can be viewed as a sequence of increasingly selective developmental steps (induction→commitment→rescue) that are signaled by αβTCR engagements of intrathymic ligands.

Introduction

Positive selection is the process by which immature αβTCR thymocytes are induced to differentiate into phenotypically mature $CD4^+8^-$ or $CD8^+4^-$ (single-positive [SP]) T cells (von Boehmer et al., 1993; Robey and Fowlkes, 1994; Kisielow and von Boehmer, 1995; Marrack and Kappler, 1997). This process involves lineage commitment, maturation, and the acquisition of immunocompetence, and these events may occur either sequentially or concurrently (Janeway, 1988; Chan et al., 1993). The specificity of αβTCR expressed on immature thymocytes is critical in determining the outcome of positive selection in that only thymocytes expressing αβTCR with appropriate specificity for intrathymic self-ligands fulfill the developmental program to become mature SP T cells (Singer et al., 1986; Kisielow et al., 1988; Teh et al., 1988; Scott et al., 1989). However, the developmental steps involved in positive selection and signaled by αβTCR remain uncertain, as precursor-progeny relationships among thymocyte subpopulations have not been fully clarified (Guidos et al., 1989; Shortman et al., 1990; Kisielow and von Boehmer, 1995).

By use of an intrathymic adoptive transfer system to assess the developmental potential of thymic subpopulations (Goldschneider et al., 1986), DP thymocytes were found to be the immediate precursors of $CD4^+$ and $CD8^+$ SP T cells (Guidos et al., 1989). However, as most DP thymocytes fail to mature into SP T cells (Egerton et al., 1990; Huesmann et al., 1991; Lucas et al., 1993; Marodon and Rocha, 1994), an understanding of the developmental progression involved in positive selection required identification of the DP thymocyte subsets that contained SP T cell precursors. High surface expression of CD69 and CD5 has been found to delineate a small subset of DP thymocytes enriched in SP T cell precursors (Yamashita et al., 1993; Punt et al., 1996; Anderson et al., 1997). More recently, maintenance of c-Kit surface expression has been used to identify a DP thymocyte subpopulation that is similarly enriched in SP T cell precursors (Akashi and Weissman, 1996; Akashi et al., 1998). Thus, DP thymocytes are heterogenous, with certain subpopulations selectively enriched in thymocytes that have been signaled to commit to either the $CD4^+$ or $CD8^+$ SP T cell lineage ("lineage commitment") and to differentiate into long-lived SP T cells ("rescue").

In the present study, we wished to determine if there existed an even earlier positive selection step signaled by αβTCR that preceded lineage commitment. Immature thymocytes first rearrange their TCRβ locus at the $CD4^-8^-$ (DN) stage of development to express surface pre-TCR complexes, which signal the cells to differentiate into DP thymocytes and to begin TCRα rearrangement (Mombaerts et al., 1992; Groettrup and von Boehmer, 1993; Groettrup et al., 1993; von Boehmer and Fehling, 1997). As a result, complete αβTCR surface complexes are first expressed on $CD5^{lo}$ DP thymocytes. Even though upregulation of CD5 expression is only signaled by surface TCR/CD3 complexes, it has not been formally demonstrated that $CD5^{lo}$ DP thymocytes differentiate into $CD5^{hi}$ DP thymocytes in vivo and that they require TCR signals to do so. We therefore assessed if $CD5^{lo}$ DP cells are precursors of $CD5^{hi}$ DP thymocytes in vivo and further assessed if this developmental transition was a selection step signaled by αβTCR. Concurrently, we also examined the relationship of this early αβTCR signaling step to lineage commitment. The result of the present study is the identification of an early step in positive selection ("induction") that is likely to be the first selection step mediated by αβTCR. We demonstrate that the induction step fixes αβTCR specificity but does not induce lineage commitment. Rather, we found that lineage commitment occurs after induction, as does negative selection of Vβ-specific TCR deletions induced by endogenous superantigens. Thus, the present study has important implications for our understanding of positive selection and repertoire selection in the thymus.

Results

The present study was undertaken to determine if there exists an αβTCR-mediated selection step prior to lineage commitment in the thymus. To identify and isolate

*To whom correspondence should be addressed (e-mail: singera@mail.nih.gov).
†Present address: Department of Biology, Haverford College, Haverford, Pennsylvania 19041.

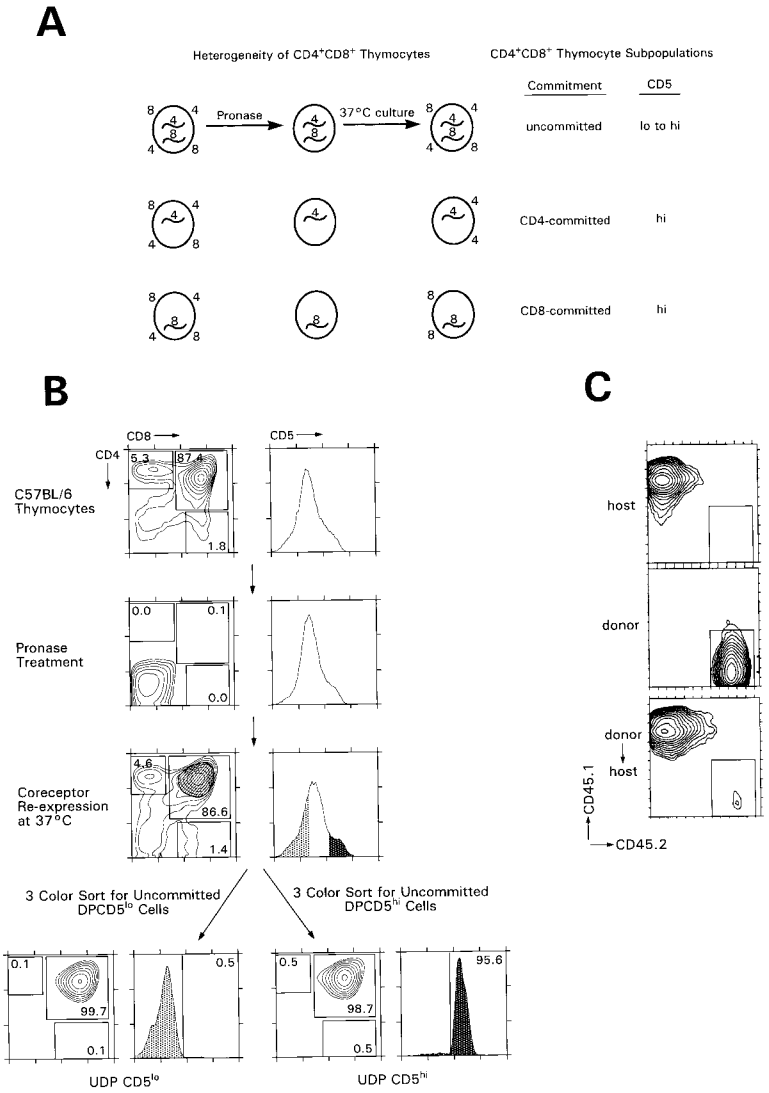

Figure 1. Identification, Isolation, and Intrathymic Transfer of Lineage-Uncommitted $CD4^+CD8^+$ Thymocytes Detected by the Coreceptor Reexpression Assay

(A) Lineage-uncommitted and lineage-committed DP thymocytes can be distinguished by the coreceptor reexpression assay in which thymocytes are treated with pronase to remove preexisting coreceptor molecules and then cultured overnight at 37°C to allow them to reexpress the coreceptor proteins they are actively synthesizing. Cells that reexpress both CD4 and CD8 proteins are defined as lineage-uncommitted DP thymocytes, while cells that reexpress only one coreceptor are considered to be lineage-committed thymocytes. Lineage-uncommitted DP thymocytes are themselves heterogenous for CD5 expression, with both $CD5^{lo}$ and $CD5^{hi}$ subpopulations (Punt et al., 1996). Lineage-committed DP thymocytes are uniformly $CD5^{hi}$ (Punt et al., 1996).

(B) Isolation of $CD5^{lo}$ and $CD5^{hi}$ uncommitted DP thymocytes. Whole B6 thymocytes were pronase stripped and cultured overnight at 37°C. Cells reexpressing both CD4 and CD8 are termed uncommitted double positive (UDP) thymocytes. After coreceptor reexpression culture, three-color sorting was used to purify UDP $CD5^{lo}$ and UDP $CD5^{hi}$ subpopulations. UDP $CD5^{lo}$ thymocytes were >99% pure, and UDP $CD5^{hi}$ thymocytes were >98% pure.

(C) Identification of donor-derived thymocytes after intrathymic injection. Donor-derived (CD45.2) thymocytes that had been injected into recipient (CD45.1) thymi were unambiguously identified in the recipient thymus as $CD45.1^-CD45.2^+$ cells.

DP thymocytes that have not undergone lineage commitment and so are actively synthesizing both coreceptor molecules, we treated whole thymocyte populations with the extracellular protease pronase to remove preexisting coreceptor molecules from the cell surface (Figure 1A). Placement of the stripped cells into overnight 37°C suspension culture results in reexpression of only those coreceptor molecules that the individual cells are actively synthesizing, as reexpression of CD4 and CD8 coreceptor molecules requires both active transcription and protein synthesis (Suzuki et al., 1995). While lineage-committed DP thymocytes reexpress only one coreceptor molecule after pronase stripping and so appear $CD4^+8^-$ or $CD4^-8^+$ after overnight culture, lineage-uncommitted DP thymocytes reexpress both CD4 and CD8 coreceptor molecules after pronase stripping and so appear DP after overnight culture (Figure 1A).

Interestingly, uncommitted DP thymocytes are a heterogenous cell population with regard to CD5 expression in that 93%–95% of uncommitted DP thymocytes are $CD5^{lo}$ and 5%–7% are $CD5^{hi}$ (Punt et al., 1996). CD69 expression identifies similar subpopulations of uncommitted DP thymocytes in that $CD5^{lo}$ uncommitted DP thymocytes are also $CD69^{lo}$, and $CD5^{hi}$ uncommitted DP thymocytes are also $CD69^{hi}$ (Punt et al., 1996). In the present study, we have utilized CD5 expression to distinguish subpopulations of uncommitted DP thymocytes because CD5 expression, unlike CD69 expression, is resistant to pronase stripping (Kearse et al., 1995; Punt et al., 1996). As CD5 expression is upregulated on thymocytes and T cells in response to TCR signals (Kearse et al., 1995), the existence of $CD5^{hi}$ uncommitted DP thymocytes suggested the possibility of an early TCR-signaled selection step that increased CD5 expression without inducing lineage commitment. However, it was also possible that $CD5^{lo}$ and $CD5^{hi}$ subpopulations of uncommitted DP thymocytes represented independently arising and unrelated developmental pathways in the thymus.

In the present study, we isolated purified populations of $CD5^{lo}$ and $CD5^{hi}$ uncommitted DP thymocytes by electronically sorting lineage-uncommitted DP thymocytes (as determined by the coreceptor reexpression assay) into $CD5^{lo}$ and $CD5^{hi}$ subpopulations by three-color flow cytometry (Figure 1B). To examine the developmental potential of $CD5^{lo}$ and $CD5^{hi}$ uncommitted DP thymocyte

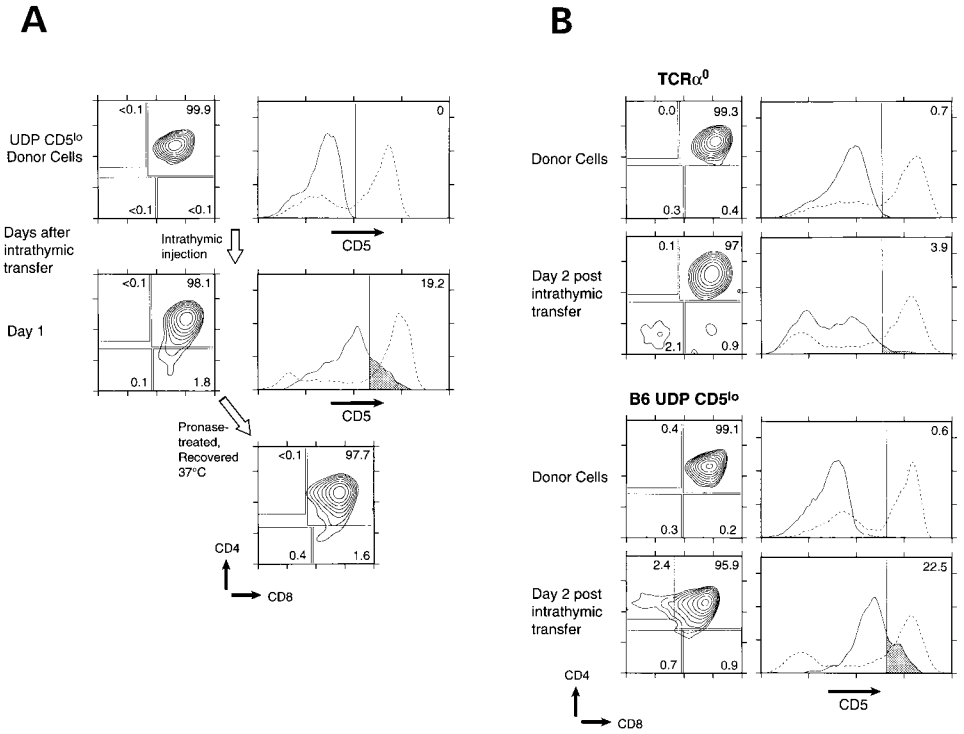

Figure 2. Precursor-Progeny Relationship between Uncommitted DP CD5^{lo} and Uncommitted DP CD5^{hi} Thymocytes that Is Signaled by αβTCR

(A) Sorted uncommitted DP (UDP) CD5^{lo} thymocytes from B6 (CD45.2) mice were intrathymically injected into unirradiated B6-congenic (CD45.1) recipients. One day later, thymocytes from inoculated mice were assessed by multi-color flow cytometry. Expression of CD4, CD5, and CD8 on donor thymocytes is displayed. In the CD5 histograms, the solid line represents CD5 expression on donor thymocytes; the dashed line represents CD5 expression on CD8$^-$ thymocytes from normal B6 mice and is used as a control to define CD5^{lo} and CD5^{hi} surface expression. CD5^{hi} donor thymocytes are indicated by shading. Thymocytes from inoculated mice were pronase stripped and cultured overnight. CD45 is resistant to pronase stripping, allowing us to examine donor-derived CD45.1$^-$CD45.2$^+$ thymocytes in the coreceptor reexpression assay (bottom profile).

(B) αβTCR is required for differentiation of uncommitted DP CD5^{lo} thymocytes into CD5^{hi} cells. Uncommitted DP (UDP) CD5^{lo} thymocytes from TCRα^o (CD45.2) and B6 (CD45.2) mice were intrathymically injected into congenic (CD45.1) recipients. Two days later, donor-derived cells were assessed for expression of CD4, CD5, and CD8. In the CD5 histograms, the solid line represents CD5 expression on donor thymocytes; the dashed line represents CD5 expression on CD8$^-$ thymocytes from normal B6 mice, and is used as a control to define CD5^{lo} and CD5^{hi} surface expression. CD5^{hi} donor thymocytes are indicated by shading.

subsets as well as their potential interrelationship, we injected each cell type directly into the thymi of unirradiated host mice so that the injected thymocytes could continue to differentiate within a normal thymic environment. The progeny of injected donor thymocytes were distinguished from resident host thymocytes by expression of the CD45 allelic marker such that donor thymocytes were CD45.1$^-$CD45.2$^+$ whereas host thymocytes were CD45.1$^+$CD45.2$^-$ (Figure 1C).

Precursor-Progeny Relationship between CD5^{lo} and CD5^{hi} Uncommitted DP Thymocytes Signaled by αβTCR

To determine if uncommitted DP CD5^{lo} thymocytes differentiate into uncommitted DP CD5^{hi} thymocytes, we injected purified uncommitted DP CD5^{lo} thymocytes into the thymi of host mice. One day post transfer, the injected thymocytes still remained DP, but 19% had upregulated CD5 expression to the high level expressed on SP T cells (Figure 2A). To determine if these newly arising CD5^{hi} DP thymocytes still remained lineage uncommitted, they were pronase stripped and cultured overnight at 37°C. Essentially all the cells reexpressed both CD4 and CD8 (Figure 2A), indicating that they were still actively synthesizing both coreceptor molecules and so remained lineage uncommitted. To determine if an intrathymic αβTCR signal was required for CD5^{lo} uncommitted DP thymocytes to upregulate CD5 expression, we isolated and transferred CD5^{lo} uncommitted DP thymocytes from TCRα^o mice whose thymocytes cannot express αβTCR (Mombaerts et al., 1992). Unlike donor CD5^{lo} uncommitted DP thymocytes from normal mice that upregulated CD5 expression within 1 day after transfer, CD5^{lo} uncommitted DP thymocytes from TCRα^o mice did not upregulate CD5 expression even 2 days after intrathymic transfer (Figure 2B). The very few TCRα^o cells that appear to have upregulated CD5 expression were not consistently observed. In comparison, CD5^{lo} uncommitted DP thymocytes from normal B6 mice were not only giving rise to CD5^{hi} progeny, but, by day 2 after transfer, were also giving rise to a few CD4$^+$8$^-$ SP progeny (Figure 2B).

We conclude that CD5^{lo} uncommitted DP thymocytes give rise to CD5^{hi} lineage-uncommitted progeny and that this developmental step is signaled in the thymus by αβTCR. That only about 20% of CD5^{lo} uncommitted DP

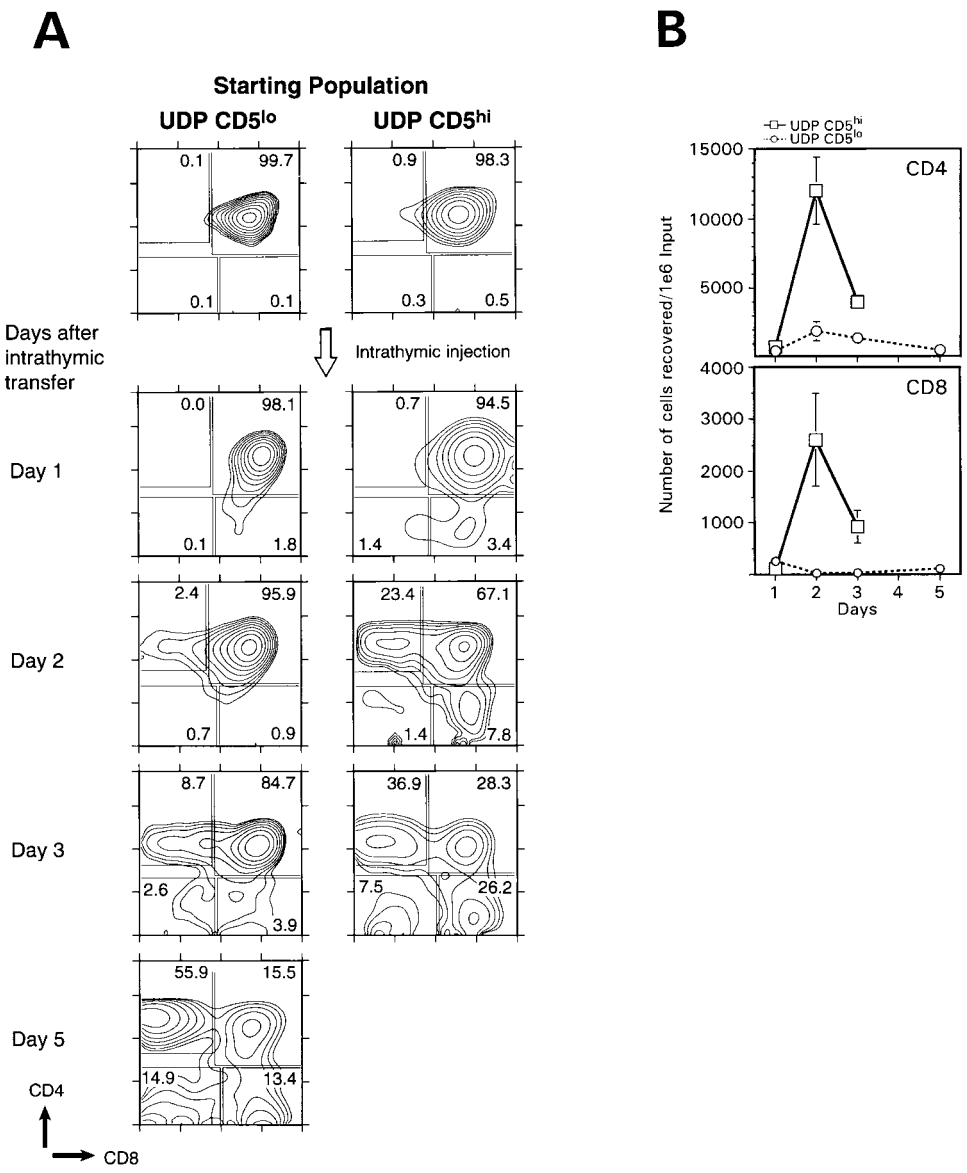

Figure 3. Both CD5^{lo} and CD5^{hi} Uncommitted DP Thymocyte Subpopulations Give Rise to SP T Cell Progeny upon Intrathymic Transfer, but They Do So with Different Kinetics

Uncommitted DP (UDP) CD5^{lo} and uncommitted DP CD5^{hi} cells from B6 (CD45.2) mice were intrathymically injected into congenic (CD45.1) recipient mice. The recipient thymi were analyzed by multi-color flow cytometry at the indicated times post transfer (A). Donor-derived cells were gated to be CD45.1^{-}CD45.2^{+}, and expression of CD4 versus CD8 on these donor-derived cells is shown (A). The numbers of donor-derived CD4 and CD8 SP progeny that were recovered from thymi inoculated with either UDP CD5^{lo} (circle) or UDP CD5^{hi} (square) thymocyte subpopulations were determined at the indicated times post transfer and normalized per 10^6 donor thymocytes injected (B).

thymocytes were observed to become CD5^{hi} uncommitted DP thymocytes was consistent with this step being a selection step, as only a minority of $\alpha\beta$TCR would be expected to have specificity for intrathymic ligands and therefore to signal CD5 upregulation.

If TCR signaling of CD5^{lo} uncommitted DP thymocytes to become CD5^{hi} uncommitted DP thymocytes were in fact an early selection step, CD5^{hi} uncommitted DP thymocytes should be developmentally more advanced and so should generate detectable SP progeny more quickly than CD5^{lo} uncommitted DP thymocytes. Consequently, we isolated CD5^{lo} and CD5^{hi} uncommitted DP thymocytes from normal mice, injected them into the thymi of CD45.1 recipient mice, and phenotyped them at different times after transfer (Figure 3A). As predicted, both CD5^{lo} and CD5^{hi} uncommitted DP thymocytes gave rise to detectable SP progeny whose frequencies increased with time after transfer. Indeed, CD5^{lo} uncommitted DP thymocytes lagged behind CD5^{hi} uncommitted DP thymocytes by 1–2 days in giving rise to significant frequencies of CD4^{+} and CD8^{+} SP progeny (Figure 3A). The SP progeny that were generated after intrathymic transfer resembled normal SP thymocytes in their high-level expression of TCRβ and CD5 (data not shown). It might also be noted that CD4^{+} SP progeny of uncommitted DP CD5^{lo} thymocytes appeared earlier

than CD8+ SP progeny, as they do during normal ontogeny.

If differentiation to the CD5hi uncommitted DP stage of development initially identifies thymocytes expressing selectable αβTCR, CD5hi uncommitted DP thymocytes should be significantly enriched in cells capable of maturing into SP T cells. Consequently, CD5hi uncommitted DP should give rise to many more SP T cells than CD5lo uncommitted DP thymocytes. As predicted, we found that uncommitted DP CD5hi thymocytes generated 6–20 times more CD4+ and CD8+ SP progeny than did the same number of uncommitted DP CD5lo thymocytes (Figure 3B).

We conclude that there exists a precursor-progeny relationship between CD5lo and CD5hi uncommitted DP thymocytes such that CD5lo uncommitted DP thymocytes differentiate into CD5hi uncommitted DP thymocytes in response to αβTCR signals. Thus, only DP thymocytes that have successfully rearranged both TCRα and TCRβ gene loci and expressed αβTCR with presumed specificity for intrathymic ligands are signaled to become CD5hi uncommitted DP thymocytes. As a result, differentiation of CD5lo into CD5hi uncommitted DP thymocytes represents the initial selection step in the identification of thymocytes expressing appropriate αβTCR.

Downregulation of RAG1 and RAG2 upon Differentiation of CD5lo Uncommitted DP Thymocytes into CD5hi Uncommitted DP Thymocytes

Fixation of αβTCR specificity results from downregulation of RAG1 and RAG2 expression and is known to occur in response to αβTCR signals during positive selection of DP thymocytes into SP T cells (Turka et al., 1991; Borgulya et al., 1992; Takahama and Singer, 1992; Brandle et al., 1994). In fact, DP CD5hi thymocytes are known to have reduced numbers of RAG transcripts (Sheard et al., 1996), but it is unknown whether these cells have already undergone lineage commitment or not. Thus, it was conceivable that downregulation of RAG1 and RAG2 occurred prior to lineage commitment and was the result of the initial αβTCR signals that induced uncommitted DP CD5lo thymocytes to become uncommitted DP CD5hi thymocytes. To examine this possibility, we compared CD5lo and CD5hi uncommitted DP thymocytes for expression of RAG1 and RAG2 transcripts (Figure 4). Interestingly, RAG1 and RAG2 expression was 10-fold greater in CD5lo uncommitted DP thymocytes than in their CD5hi progeny as determined by semiquantitative RT-PCR with normalization to β_2-microglobulin expression (Figure 4). Consequently, initial αβTCR selection signals that induce CD5lo uncommitted DP thymocytes to become CD5hi uncommitted DP thymocytes also fix their αβTCR specificity by downregulating RAG1 and RAG2 expression without inducing lineage commitment.

The MHC Specificity of the Initial αβTCR Selection Step Does Not Dictate but Does Skew the Developing Thymocyte's Ultimate Lineage Choice

Since CD5lo uncommitted DP thymocytes are signaled to differentiate into CD5hi uncommitted DP thymocytes

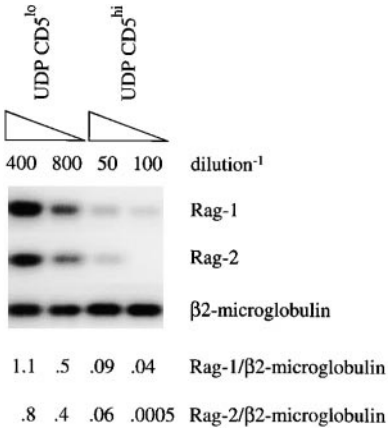

Figure 4. Downregulation of RAG1 and RAG2 mRNA Transcripts in Uncommitted DP CD5hi Cells

Serial dilutions of cDNA from sorted uncommitted DP (UDP) CD5lo and uncommitted DP CD5hi populations were PCR amplified, electrophoresed on agarose gels, transferred to nylon membranes, and hybridized with the indicated probes. Dilution ranges of cDNA were chosen to give equivalent β_2-microglobulin amplification. The ratio of RAG1 or RAG2 product to control β_2-microglobulin product is indicated at the bottom. The 10-fold difference in RAG expression between CD5lo and CD5hi uncommitted DP thymocytes may be an underestimate, as extracts from uncommitted DP CD5lo thymocytes were diluted 8-fold more than those from uncommitted DP CD5hi cells in order to detect comparable β_2-microglobulin signals in both cell extracts. As a result, without normalization to β_2-microglobulin expression, RAG1 and RAG2 expression in CD5lo uncommitted DP precursors was 80-fold greater than in their CD5hi uncommitted DP progeny.

by αβTCR signals generated by interaction with intrathymic ligands, it was possible that this initial selection step, by fixing the developing thymocytes' TCR specificities, also dictated their ultimate lineage fate. To assess such a possibility, we reasoned that uncommitted DP thymocytes from MHC class II–deficient mice became CD5hi by expressing αβTCR specific for MHC class I–presented ligands, whereas uncommitted DP thymocytes from MHC class I–deficient mice became CD5hi by expressing αβTCR specific for MHC class II–presented ligands. To ask whether the MHC specificity of this initial αβTCR interaction determined the ultimate lineage fate of the developing thymocyte, we purified CD5hi uncommitted DP thymocytes from either MHC class I–deficient or MHC class II–deficient mice and injected them into the thymi of normal recipient mice (Figure 5). Interestingly, CD5hi uncommitted DP thymocytes from both MHC class I–deficient and MHC class II–deficient strains gave rise to both CD4+ and CD8+ SP progeny (Figure 5), demonstrating that the lineage fate of uncommitted DP thymocytes was not dictated by the MHC specificity of their initial αβTCR interaction. Nevertheless, it is also clear that the MHC specificity of the initial αβTCR interaction did skew their ultimate lineage choice (Figure 5). That is, following transfer into normal thymi, CD5hi uncommitted DP thymocytes from MHC class II–deficient mice gave rise to mostly CD8+ SP progeny, consistent with the MHC class I specificity of their initial selection step; and CD5hi uncommitted DP thymocytes from MHC class I–deficient mice gave rise to mostly CD4+ SP progeny, consistent with the MHC class II

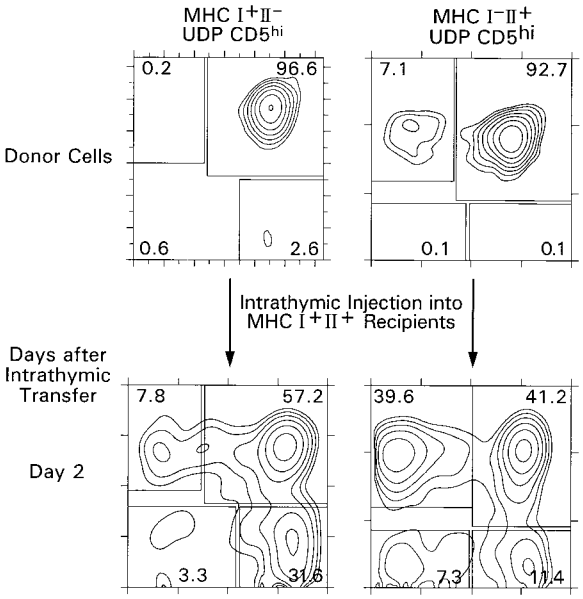

Figure 5. The Specificity of the Initial TCR Interaction Leading to CD5 Upregulation Does Not Dictate the Lineage Fate of the DP Cell

Uncommitted DP (UDP) CD5^{hi} thymocytes from either MHC class II–deficient or β_2-microglobulin-deficient mice were injected into congenic (CD45.1) recipients that expressed both MHC class I and class II molecules. The phenotype of the adoptively transferred cells was determined 2 days post transfer.

specificity of their initial selection step. Thus, the specificity of the initial selection step skews but does not fix the ultimate lineage fate of the DP thymocyte.

Lineage Commitment Occurs after the Initial αβTCR Selection Step and Can Also Result in Vβ-Specific Deletion

The present data directly demonstrate a precursor-progeny relationship in the CD5^{lo} uncommitted DP→CD5^{hi} uncommitted DP induction step. In addition, these data demonstrate that CD5^{hi} uncommitted DP thymocytes become lineage committed upon further differentiation into CD4$^+$ and CD8$^+$ SP T cells. We reasoned that one way to independently demonstrate that CD5^{hi} uncommitted DP thymocytes differentiate into CD5^{hi} lineage-committed DP thymocytes was to examine Vβ usage by these different DP thymocyte subsets (Guidos et al., 1990). To compare Vβ usage by uncommitted and lineage-committed DP thymocytes, we sorted for CD4$^+$8^{lo} DP thymocytes because this subpopulation is unique in containing both CD4-committed and CD8-committed DP thymocytes, as well as lineage-uncommitted DP thymocytes (Suzuki et al., 1995). After pronase stripping and overnight culture, recovered thymocytes were phenotyped by four-color flow cytometry for CD4, CD8, CD5, and Vβ expression. In this way, four distinct DP thymocyte subpopulations were simultaneously identified and assessed for Vβ expression: uncommitted CD5^{lo} DP thymocytes, uncommitted CD5^{hi} DP thymocytes, CD4-committed CD5^{hi} DP thymocytes, and CD8-committed CD5^{hi} DP thymocytes.

Vβ6 is deleted in Mls-1^a mice and appears only in SP T cells from Mls-1^b strains (Kappler et al., 1988; MacDonald et al., 1988). Vβ6 frequencies were comparable in both CD5^{lo} and CD5^{hi} uncommitted DP thymocytes from Mls-1^a versus Mls-1^b mice (Figure 6). However, the frequency of Vβ6 cells abruptly declined in CD5^{hi} lineage-committed DP thymocytes from Mls-1^a mice but not Mls-1^b mice, revealing that Vβ6 cells are deleted in Mls-1^a strains after they are signaled to become CD5^{hi} uncommitted cells and prior to, or concurrent with, their being signaled to undergo lineage commitment. Control Vβ8.2/3 expression was not negatively affected by Mls-1^a expression in any of the subsets examined; in fact, control Vβ8.2/3 expression in Mls-1^a mice showed compensatory increases in the lineage-committed subsets from which Vβ6 cells had been deleted (Figure 6). These results confirm that the sequence for positive selection of αβTCR DP thymocytes is CD5^{lo} uncommitted DP thymocytes→CD5^{hi} uncommitted DP thymocytes→lineage committed DP thymocytes and demonstrate that not all uncommitted DP CD5^{hi} cells will mature into lineage-committed and SP T cells, as some of these uncommitted DP CD5^{hi} cells are destined for the alternative fate of negative selection.

Discussion

The present study demonstrates the existence of an early positive selection step signaled by αβTCR that terminates RAG expression but precedes lineage commitment in the thymus. For thymocytes expressing an αβTCR that can engage intrathymic MHC/peptide ligands, fixation of αβTCR specificity occurs prior to and independently of ultimate cell fate determinations. In addition, the present study formally documents a precursor-progeny relationship between CD5^{lo} and CD5^{hi} uncommitted DP thymocytes and shows that CD5^{hi} uncommitted DP thymocytes can either be positively selected for further differentiation into SP T cells or can be negatively selected to undergo cell death.

The goal of the present study was to identify the initial positive selection step signaled by surface αβTCR complexes and to determine if such an initial positive selection step preceded lineage commitment. To do so, we identified lineage-uncommitted DP thymocytes by their active synthesis of both CD4 and CD8 molecules in the coreceptor reexpression assay. That such DP thymocytes were in fact lineage uncommitted was shown by the fact that (1) they continued to actively synthesize both coreceptor molecules for 1–2 additional days after intrathymic transfer, (2) they retained their potential to differentiate into either CD4$^+$ or CD8$^+$ T cells, and (3) they expressed an "immature" TCR repertoire containing potentially autoreactive TCR-Vβ specificities that were absent from the "mature" repertoire expressed by lineage-committed DP thymocytes.

During intrathymic differentiation of normal (i.e., non-TCR transgenic) thymocytes, complete αβTCR complexes are not expressed before the DP stage of differentiation (Groettrup and von Boehmer, 1993; Groettrup et al., 1993; Shores et al., 1993; von Boehmer et al., 1993; von Boehmer and Fehling, 1997). It is at the CD4$^-$8$^-$ stage of development that thymocytes first rearrange their TCRβ locus and express surface pre-T complexes that signal the cells to differentiate into DP thymocytes ("β-selection") and to begin TCRα rearrangement (Abraham et al., 1991; Molina et al., 1992;

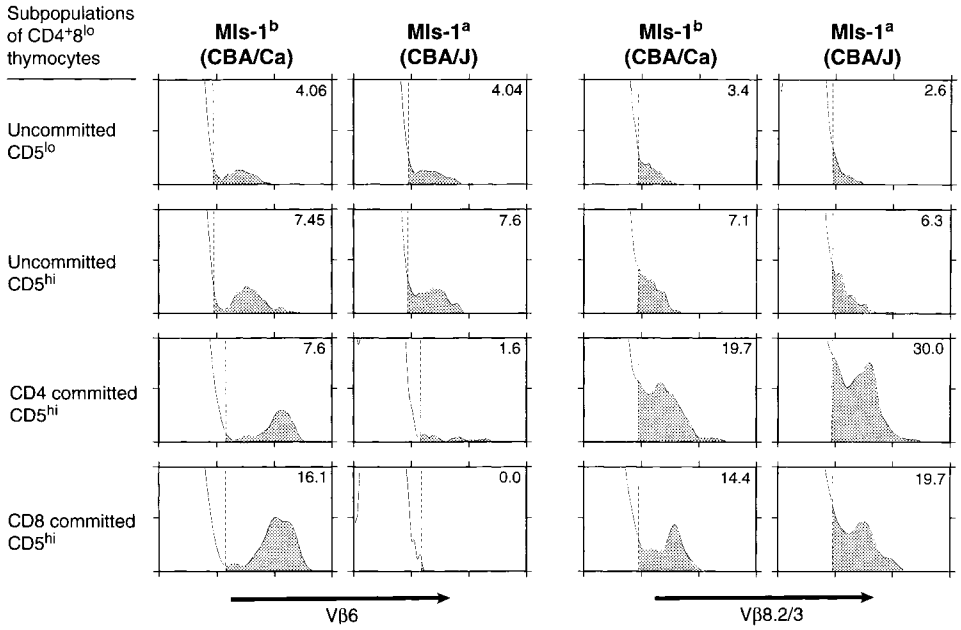

Figure 6. Negative Selection by Endogenous Superantigens Occurs as Uncommitted CD5^{hi} DP Thymocytes Undergo Lineage Commitment

Thymocytes from either CBA/Ca (Mls-1^{b}) or CBA/J (Mls-1^{a}) mice were sorted to be CD4^{+}8^{lo}, pronase stripped, and cultured overnight at 37°C. The CD4^{+}8^{lo} DP subset contains both lineage-committed and lineage-uncommitted DP subpopulations (Suzuki et al., 1995). After pronase stripping and overnight culture, we identified lineage-uncommitted thymocytes by their reexpression of both CD4 and CD8 coreceptors; in contrast, lineage-committed cells reexpressed only one coreceptor and were uniformly CD5^{hi}. Multi-color flow cytometry was used to determine Vβ expression on the DP thymocyte subpopulations as indicated.

Groettrup and von Boehmer, 1993; Groettrup et al., 1993; Levelt et al., 1993; Mombaerts et al., 1994; von Boehmer and Fehling, 1997). As a result, complete αβTCR surface complexes are first expressed on CD5^{lo} uncommitted DP thymocytes. In the present study, we found that differentiation of CD5^{lo} uncommitted DP thymocytes into CD5^{hi} uncommitted DP thymocytes requires αβTCR signals that are presumably elicited in the thymus by engagement of intrathymic ligands. Thus, the ability of CD5^{lo} uncommitted DP thymocytes to be signaled to differentiate into CD5^{hi} uncommitted DP thymocytes is dependent upon their expression of a selectable αβTCR. CD5^{lo} uncommitted DP thymocytes that do not successfully rearrange their TCRα locus and fail to express αβTCR surface complexes would not be signaled to differentiate into CD5^{hi} uncommitted DP thymocytes, nor would CD5^{lo} uncommitted DP thymocytes with αβTCR that were unable to bind intrathymic ligands. Thus, differentiation of CD5^{lo} uncommitted DP thymocytes into CD5^{hi} uncommitted DP thymocytes is likely to be the initial selection step that is signaled by αβTCR.

We were surprised to find that RAG expression was downregulated in CD5^{hi} uncommitted DP thymocytes prior to their undergoing lineage commitment. However, the TCRα gene locus is not allelically excluded (Borgulya et al., 1992) so that productive TCRα rearrangements remain at risk of excision by subsequent rearrangements so long as RAG proteins are present (Petrie et al., 1993). Downregulation of RAG1 and RAG2 mRNA by initial αβTCR signals minimizes the possibility of further gene rearrangements and essentially fixes the specificity of surface αβTCR complexes. Indeed, it makes sense that DP thymocytes downregulate RAG expression to fix αβTCR specificity prior to undergoing lineage commitment because it is the MHC specificity of their αβTCR that ultimately dictates their commitment to differentiate into either a CD4^{+} or CD8^{+} T cell.

Our observation that CD5^{hi} uncommitted DP thymocytes can differentiate into either CD4^{+} or CD8^{+} SP T cells bears further comment. In MHC class I–deficient mice, CD5^{hi} uncommitted DP thymocytes would have been generated by αβTCR engagement of MHC class II–presented ligands, whereas, in MHC class II–deficient mice, CD5^{hi} uncommitted DP thymocytes would have been generated by αβTCR engagement of MHC class I–presented ligands. Upon transfer into a normal thymus expressing both MHC class I and II molecules, most CD5^{hi} uncommitted DP thymocytes obtained from MHC-deficient mice differentiated into SP T cells with a phenotype that was appropriate to the MHC specificity of their initial αβTCR interaction. However, significant numbers of CD5^{hi} uncommitted DP thymocytes differentiated into SP T cells with a phenotype that was "inappropriate" to the MHC specificity of their initial interaction. These observations confirm that CD5^{hi} uncommitted DP thymocytes are not yet fixed in their lineage specificity, even though they have downregulated RAG expression. In addition, these results also indicate that the stringency of this initial induction step is significantly less than that of the final positive selection (rescue) step that requires strict concordance between MHC specificity of the αβTCR and coreceptor phenotype.

It is interesting to consider that CD5 functions as a negative regulator of αβTCR signaling and is not simply a convenient marker of intrathymic differentiation (Tarakhovsky et al., 1995). Consequently, CD5 upregulation

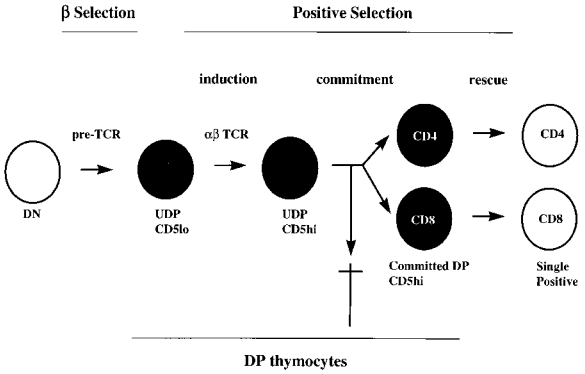

Figure 7. Proposed Developmental Sequence for Positive Selection of αβTCR Cells

The earliest thymocyte precursors are DN. DN thymocytes that successfully rearrange and express TCRβ become DP CD5^{lo} thymocytes (β-selection), and these are the first cells in the thymus to rearrange and express the clonotypic αβTCR. Only CD5^{lo} DP thymocytes with αβTCR that successfully engage intrathymic ligands are signaled to mature into CD5^{hi} DP thymocytes (induction). αβTCR signals that increase CD5 expression on uncommitted DP thymocytes also downregulate RAG1 and RAG2 expression, fixing αβTCR specificity. Subsequent signals in CD5^{hi} uncommitted DP thymocytes induce lineage commitment or, alternatively, clonal deletion. Lineage-committed CD5^{hi} DP thymocytes are short lived and require an additional αβTCR signal to become long-lived SP T cells (rescue). In our view, positive selection is the result of a linear developmental progression within the DP thymocyte population that is signaled by αβTCR: induction→commitment→rescue. Each of these steps is progressively more stringent, perhaps in part because CD5, whose expression increases during positive selection, functions as a negative regulator of αβTCR signaling (Tarakhovsky et al., 1995).

by CD5^{lo} uncommitted DP thymocytes during the induction step dampens subsequent αβTCR selection signals in CD5^{hi} uncommitted DP thymocytes. Thus, as thymocytes increase CD5 expression, the generation of active αβTCR differentiative signals would require quantitatively increased numbers of αβTCR to be engaged by intrathymic ligands. As a result, the selection criteria for αβTCR become increasingly stringent as CD5 expression increases.

The simplest interpretation of the present study views positive selection as a multistep process leading to the differentiation of CD5^{lo} uncommitted DP thymocytes into long-lived, phenotypically mature CD4$^+$ or CD8$^+$ T cells (Figure 7). First, CD5^{lo} uncommitted DP thymocytes expressing an αβTCR able to bind intrathymic ligands are signaled to upregulate CD5 expression and to differentiate into CD5^{hi} uncommitted DP thymocytes (induction). Second, selected uncommitted DP CD5^{hi} cells are signaled to undergo lineage commitment and to differentiate into lineage-committed CD5^{hi} DP thymocytes (commitment). While αβTCR signals are a component of the lineage commitment decision, additional signals may also be involved, but their identity remains highly controversial (Borgulya et al., 1991; Robey et al., 1991; Chan et al., 1993; Davis et al., 1993; Suzuki et al., 1995). Third, lineage-committed CD5^{hi} DP thymocytes are thought to be short-lived cells that require a TCR-induced rescue signal to differentiate into long-lived SP T cells (rescue). In the absence of such a rescue signal, lineage-committed DP would not be expected to survive long enough for inappropriate coreceptors to disappear from their cell surface and so would fail to appear as phenotypically SP T cells. Thus, we think there are at least three discrete TCR signaling steps involved in the positive selection of αβTCR$^+$ DP thymocytes: induction, commitment, and rescue (Figure 7). It is in the initial induction step that selectable αβTCR are first identified and their receptor specificity fixed by downregulation of RAG expression. It is after αβTCR specificity is fixed that TCR-specific cell fate determinations (clonal elimination versus lineage commitment and rescue) are made.

We think that αβTCR signals are required for each positive selection step and that αβTCR specificity is assessed with increasing stringency at each developmental transition. Indeed, it has recently been demonstrated that progressively more mature thymocyte populations are increasingly enriched in a positively selected αβTCR specificity (Sant'Angelo et al., 1998). In the present study, we found that CD5^{hi} uncommitted DP thymocytes gave rise to six times as many CD4$^+$ and 20 times as many CD8$^+$ SP progeny than did their CD5^{lo} uncommitted DP precursors, revealing that uncommitted DP CD5^{hi} thymocytes are enriched in selectable TCRs. However, we also found that not all uncommitted DP CD5^{hi} thymocytes are destined to mature into SP T cells, as some of them will be negatively selected (Shortman et al., 1991). Indeed, our present data identify the commitment step involving the differentiation of uncommitted DP CD5^{hi} DP thymocytes into lineage-committed cells as one step in which αβTCR specificities can be negatively selected and removed from the αβTCR repertoire.

An adoptive transfer approach similar to the one used in the present study was utilized to first document that DP thymocytes were the precursors of SP T cells (Guidos et al., 1989). However, only large DP thymocytes that were presumed to be "blasts" were initially found to yield SP progeny (Guidos et al., 1989), but a more recent assessment revealed that both small and large DP thymocytes can differentiate into SP progeny, albeit with markedly different efficiencies (Lundberg and Shortman, 1994; Swat et al., 1994). In the present study, CD5^{hi} uncommitted DP thymocytes are larger than CD5^{lo} uncommitted DP thymocytes as assessed by forward light scatter, but are smaller than mature SP T cells (data not shown).

Our present conclusions contrast with the recent suggestion that at least two different positive selection pathways exist for generation of SP T cells. In a recent study, c-Kit^{lo} and c-Kit^{hi} thymocyte subsets were both found to give rise to SP T cells, albeit with differing efficiencies (c-Kit^{hi} thymocytes being much more efficient) (Akashi and Weissman, 1996; Akashi et al., 1998). It was suggested that c-Kit^{lo} and c-Kit^{hi} thymocytes differentiated into SP T cells by two independent positive selection pathways. To compare the present study with these previous studies, it is important to appreciate that CD5^{lo} uncommitted DP thymocytes are c-Kit^{lo}, but CD5^{hi} uncommitted DP thymocytes are not c-Kit^{hi} cells. Instead, c-Kit^{hi} cells have the phenotype of CD5^{hi} lineage-committed DP thymocytes in that they are IL-7Rα^{hi}Bcl2^{hi} (Punt et al., 1996). Thus, c-Kit^{lo} and c-Kit^{hi} thymocytes are at least two selection steps apart from one another. The present study demonstrates the existence of a transitional intermediate between these two populations (i.e.,

the CD5^{hi} uncommitted DP population) and so argues that both precursor cell types may in fact be part of a single linear pathway of positive selection.

In conclusion, the present study results in a relatively straightforward picture of positive selection in the thymus as a developmental progression that is initiated with the earliest expression of αβTCR. The present analysis represents our simplest interpretation of currently available data and suggests that positive selection can be viewed as a linear series of increasingly selective steps in the thymus that are signaled by αβTCR.

Experimental Procedures

Mice

Normal B6 (CD45.2), B6-congenic (CD45.1), CBA/Ca (Mls-1^b), and CBA/J (Mls-1^a) mice were obtained from the Frederick Cancer Research and Development Center. TCRα^o mice (Mombaerts et al., 1992) were obtained from The Jackson Laboratory. MHC class II^o (Grusby et al., 1991) and $\beta_2$microglobulin-deficient mice (Koller et al., 1990) were both crossed to the C57BL/6 background. All mice were housed in a specific pathogen-free facility and used at 4–12 weeks of age.

Isolation of Lineage-Uncommitted Double-Positive Thymocytes

Suspensions of whole thymocytes were treated with 0.01% pronase (Calbiochem Novabiochem) solution in PBS, to remove surface CD4 and CD8 coreceptors (Kearse et al., 1995; Suzuki et al., 1995). Cells were then cultured overnight at 37°C to permit coreceptor reexpression (Suzuki et al., 1995). Cell recovery after pronase treatment and 37°C overnight culture was approximately 70% of starting cell number. Cells that have recently committed to either the CD4 or CD8 lineage reexpress either the CD4 or CD8 coreceptor (Suzuki et al., 1995). Lineage-uncommitted cells are defined as cells that reexpress both CD4 and CD8 following this treatment (Suzuki et al., 1995; Punt et al., 1996). To obtain lineage-uncommitted DP thymocytes, pronased thymocytes that had been subjected to overnight culture at 37°C were stained with anti-CD4 FITC (RM4-5, Pharmingen) anti-CD5 PE (53-7.3, Pharmingen), and anti-CD8 CY5 (CT-CD8a, Caltag). CD5 expression levels are essentially unaffected by treatment with the concentrations of pronase used in this study (Kearse et al., 1995). Stained cells could therefore be sorted on a Facstar Plus (Becton Dickinson) into uncommitted DP CD5^{lo} and uncommitted DP CD5^{hi} populations. Double-positive thymocytes from TCRα^o mice were isolated by panning on anti-CD8 coated plates (Nakayama et al., 1990).

Intrathymic Injections

Sorted thymocyte populations from B6 mice (CD45.2) were injected into the thymi of unirradiated B6-congenic (CD45.1) mice as described (Goldschneider et al., 1986). 5×10^6 uncommitted DP CD5^{lo} and 5×10^5 uncommitted DP CD5^{hi} cells were resuspended in a volume of 10 μL PBS with 1% B6 mouse serum and injected intrathymically.

Analysis of Donor-Derived Cell Populations

Single cell suspensions of thymocytes from B6-congenic (CD45.1) recipient mice that had been intrathymically injected with B6 (CD45.2) donor thymocytes were assessed by four-color flow cytometry using anti-CD45.1 biotin (A20, Pharmingen) + Streptavidin Texas Red, anti-CD45.2 FITC (104, Pharmingen), anti-CD8 CY5 (CT-CD8a, Caltag), and anti-CD5 PE, anti-CD4 PE (GK1.5, Becton Dickinson), or anti-TCRβ PE (H57-597, Pharmingen) as indicated. Where indicated, thymocytes recovered from host mice were treated with 0.01% pronase for 10 min at 37°C and cultured overnight. Pronase treatment removes preexisting CD4 and CD8 coreceptors but does not affect TCRβ, CD5, or CD45 expression (Kearse et al., 1995; data not shown). Data acquisition was performed on a dual-laser FACStar Plus (Becton Dickinson). Staining with antibodies to both CD45 alleles allowed us to unambiguously identify donor-derived cells as CD45.1$^-$CD45.2$^+$ in every experiment (Figure 1C). Dead cells were excluded from analysis by propidium iodide staining and forward light scatter gating. Doublets were further excluded from analysis by the FSC-W (forward scatter width) parameter. Donor cells represented up to 1% of total thymocytes obtained from host thymi.

Frequency of Vβ-Expressing Cells

The frequency of Vβ6, Vβ8.2/3, and TCRβ expressing cells in the various developmental stages defined in this study were assessed in thymocytes from CBA/J mice (H-2^k Mls-1^a) and control CBA/Ca mice (H-2^k Mls-1^b) by four-color flow cytometry using antibodies to CD4, CD8, CD5, and TCRβ (Vβ6: RR4-7, Pharmingen; Vβ8.2/3: KT8E, Caltag; TCRβ: H57-597; Pharmingen). Uncommitted DP thymocytes were defined as DP thymocytes that reexpressed both CD4 and CD8 after recovery from pronase treatment and consisted of both CD5^{lo} and CD5^{hi} subpopulations. DP thymocytes that reexpressed only one coreceptor after recovery from pronase treatment were considered lineage-committed DP thymocytes, all of which were CD5^{hi}.

RT-PCR

We reverse transcribed total RNA prepared from 1.5×10^4 sorted uncommitted DP CD5^{lo} or uncommitted DP CD5^{hi} cells to cDNA using a poly(dT) oligonucleotide (SUPERSCRIPT II; GIBCO-BRL), so that only polyadenylated RNA transcripts were primed and reverse transcribed to cDNA. β_2-microglobulin (Sheard et al., 1996), RAG1 and RAG2 (Merkenschlager et al., 1997) primers were used for PCR amplification and probing of serially diluted cDNA. We performed 36 cycles of PCR amplification (Taq DNA polymerase kit; GIBCO-BRL) with 1 min of denaturation at 94°C, 1 min of annealing at 55°C for β_2-microglobulin and 60°C for RAG1 and RAG2, and 1 min of polymerization at 72°C. Amplified products were analyzed on 1.5% agarose gels, transferred to nylon membranes (Hybond-N$^+$; Amersham), and hybridized with the indicated probes:

β_2-microglobulin, 5′-CGCCTCACATTGAAATCCAAATGC-3′; RAG1, 5′-CACAGAAGGAGAAGGATTCCTCAG-3′; and RAG2, 5′-CCTTAA TTCAACCAGGCTTCTCAC-3′.

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