Brief Definitive Report

Asynchronous Coreceptor Downregulation after Positive Thymic Selection: Prolonged Maintenance of the Double Positive State in CD8 Lineage Differentiation Due to Sustained Biosynthesis of the CD4 Coreceptor

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Summary

In several experimental systems analyzing the generation of single positive (SP) thymocytes from double positive (DP) thymocytes, CD4 SP cells have been shown to appear before CD8 SP cells. This apparent temporal asymmetry in the maturation of CD4 SP and CD8 SP thymocytes could either be due to divergent molecular differentiation programs of the two T cell lineages, or merely to slower degradation kinetics of the CD4 protein. To study this question in unmanipulated in vivo differentiation, we developed a four-color flow cytometry protocol which identifies a recently activated TCR^{int}CD69^{pos} thymocyte population containing DP cells and early CD4 SP cells but no CD8 SP cells. We show that these TCR^{int}CD69^{pos} thymocytes represent a transitory stage in the mainstream $\alpha\beta$ T cell lineage. The precursors of the CD8 SP cells are contained in this population as incompletely selected DP cells. Moreover, we show that expression of both coreceptors in the TCR^{int}CD69^{pos} population depends on transcriptional and translational activity, thus excluding differences in turnover rates of the CD4 and CD8 proteins as the cause of the asynchrony in differentiation of the CD4 and CD8 lineages.

The development of mature CD4 single positive (SP) and CD8 SP thymocytes from CD4/CD8 double positive (DP) precursors is mediated by signaling events through the $\alpha\beta$ TCR collectively termed positive selection. In this process, a variety of phenotypic changes occur: as a result of the selection signals, the early activation marker CD69 is transiently expressed; the TCR is upregulated from intermediate expression levels (TCR^{int}) characteristic for the majority of DP cells to high expression levels (TCR^{hi}) characteristic for SP cells; of the two coreceptors, the one which fails to match the MHC restriction of the TCR is turned off, thus generating CD8 cells with MHC class I–restricted TCRs and CD4 cells with MHC class II–restricted TCRs (1–5). In several experimental systems evidence was obtained suggesting that the generation of CD4 SP and CD8 SP thymocytes is asynchronous. In fetal development, CD4 SP thymocytes appear before CD8 SP thymocytes (6). Phenotypic analysis and BrdU labeling in the adult thymus revealed that downregulation of CD8 precedes that of CD4 after positive selection (7, 8). In reaggregate thymic organ cultures (RTOC), DP TCR–negative cells generate CD4 SP cells before CD8 SP cells (9). Adoptive transfer experiments using purified adult DP subsets also demonstrated that differentiation into CD8 SP thymocytes needs longer than differentiation into CD4 SP cells (10). Utilizing a marker (F3) differentially expressed on pre-selection and post-selection DP thymocytes, we have previously ob-

served that post-selection DP cells generate both CD8 and CD4 SP cells within 48 h of RTOC, whereas pre-selection DP cells generate only CD4 SP cells under these conditions (11). Similar results have been obtained using CD69 as a marker to distinguish pre-selection from post-selection DP thymocytes (12). Thus, unless the delayed generation of CD8 SP cells was caused by the experimental manipulations, these data suggested that cells selected into the CD8 lineage exit the DP pool 2–3 d later than cells selected into the CD4 lineage.

The temporal asymmetry in CD4 and CD8 lineage development could be due to differences in turnover rates of the CD4 and the CD8 proteins, and thus perhaps be biologically insignificant. Alternatively, selection of CD4 SP and CD8 SP cells may initiate divergent molecular differentiation programs that determine developmental decisions at this checkpoint. In this report, we identify a transitory thymocyte subset in the unmanipulated adult thymus that contains late DP cells and early CD4 SP cells but no CD8 SP cells. We show that this subset contains the precursors of the CD8 SP cells still in the DP stage, maintaining expression of CD4 by active transcription and translation.

Materials and Methods

Animals. Mice of strain BALB/c and C57Bl/6 were obtained from the specific pathogen free animal facilities of the Max-
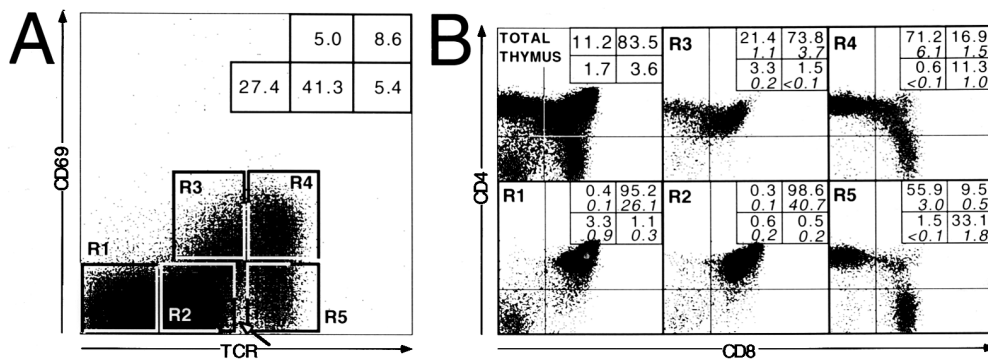
A

		5.0	8.6
	27.4	41.3	5.4

CD69 / TCR

R1, R2, R3, R4, R5

B

TOTAL THYMUS

11.2	83.5
1.7	3.6

R3

21.4	73.8
1.1	3.7
3.3	1.5
0.2	<0.1

R4

71.2	16.9
6.1	1.5
0.6	11.3
<0.1	1.0

R1

0.4	95.2
0.1	26.1
3.3	1.1
0.9	0.3

R2

0.3	98.6
0.1	40.7
0.6	0.5
0.2	0.2

R5

55.9	9.5
3.0	0.5
1.5	33.1
<0.1	1.8

CD4 / CD8

Figure 1. Thymic subsets defined by TCR levels and CD69 (*A*) and phenotypic characterization of coreceptor expression in each subset (*B*). Total thymocytes were stained for TCR, CD69, CD4 and CD8 and analyzed by four-color flow cytometry. Percentages are given in the insets, numbers in italics in *B* indicate the percentages in relation to total thymocytes. The arrow in *A* indicates the relatively empty space between R2 and R5, suggesting that passing through the CD69^{pos} gates R3 and R4 is obligatory for all cells.

Planck-Institut and were used between 4–8 wk of age unless otherwise stated.

Flow Cytometry and Monoclonal Antibodies. Analytical flow cytometry was carried out using a FACScan® or FACStar® Plus (Becton Dickinson, Mountain View, CA) and the data were processed using the Cellquest software (Becton Dickinson). The following mAb labeled with biotin (B), PE, or FITC were purchased from PharMingen (San Diego, CA): anti-CD8α (53-6.7), anti-CD4 (H129.19), anti-TCRβ (H57-597), anti-CD69 (H1.2F3), and anti-NK1.1 (PK136). RED613-labeled anti-CD8 (53-6.7) was purchased from Gibco (Eggenstein, Germany). Streptavidin (SA) conjugates used to detect biotinylated mAb were SA-RED670 (Gibco) and SA-Cy5 (Dianova, Hamburg, FRG). Cells were pre-incubated with unlabeled mAb to FcγRII/III (2.4G2) to minimize unspecific staining. Two-, three-, and four-color analyses were performed by sequential addition of the appropriate labeled mAb before adding the SA-conjugate. Stainings with individual reagents were performed for 30 min in FACS® medium (PBS containing 2% FCS) followed by washing with FACS® medium. All steps were carried out at 4°C. 2×10^5–2×10^6 cells per sample were stained and 10,000–200,000 events acquired, and then gated on viable cells using forward and sideward light scatter.

Trypsin Digestion. Thymocytes stained with anti–CD69 and anti TCR-β mAb were sorted into TCR^{int}CD69^{pos} and TCR^{hi}CD69^{pos} populations, using a FACStar®. Cell aliquots from each population ($5–10 \times 10^5$) were either incubated with 500 μl PBS or 500 μl trypsin/EDTA (Gibco) for 30 min at 37°C to cleave off the coreceptors CD4 and CD8. After the incubation, aliquots were stained for CD4 and CD8 expression. Thymocytes were kept in overnight suspension culture in 96-well flat-bottom wells (Costar, Cambridge, MA) at 2×10^5 cells/well in Iscove's medium containing 10% selected FCS. To inhibit transcription or translation, 3 μg/ml Actinomycin D (stock solution 1 mg/ml DMSO; Sigma, Deisenhofen, Germany), or 10 μg/ml CHX (stock solution 4 mg/ml PBS; Sigma), respectively, were included in the culture medium. The thymocyte recovery in the various suspension cultures was ∼20%.

Reaggregate Thymic Organ Cultures. Reaggregate thymic cultures were performed as described (9). In brief, day 15 fetal thymic lobes were treated with 1.35 mM desoxyguanosine (Sigma) for 5 d, to deplete lymphoid cells. Single cell suspensions were prepared from the remaining stroma by treatment with trypsin/EDTA (Gibco). 2.5×10^5 stroma cells were pelleted by centrifugation together with 2.5×10^5 purified TCR^{int}CD69^{pos} DP thymocytes, and the pellets were transferred onto filter discs (0.8 μm Nucleopore; Costar) floating on medium. Thymic lobes reformed

within 1 d, and were analyzed by flow cytometry after 48 h of culture. The recovery of viable cells was ∼10%.

Results

Sequential Appearance of CD4 SP and CD8 SP Thymocytes In Vivo. A number of experiments have suggested that, after positive selection, CD4 SP thymocytes develop more rapidly than CD8 SP thymocytes (6–12). With one exception (7) these studies employed previously manipulated DP cells or DP subpopulations placed in reaggregate thymic organ cultures or transferred intrathymically into syngeneic mice, so that an influence of experimental manipulations on developmental kinetics was not excluded. To detect recently generated SP thymocytes in vivo, we stained thymic cell suspensions for CD4, CD8, TCR, and CD69 expression. The early activation marker CD69 is expressed transiently on activated thymocytes and discriminates cells which have recently received a selection signal from pre-selection and late post-selection thymocytes. The stepwise increase in the level of TCR expression served as a marker for the maturation sequence. As shown in Fig. 1 *A*, this protocol defines five sequential developmental stages: R1→TCR^{neg}CD69^{neg}, R2→TCR^{int}CD69^{neg}, R3→TCR^{int}CD69^{pos}, R4→TCR^{hi}CD69^{pos}, and R5→TCR^{hi}CD69^{neg}. The obvious gap between R2 and R5 (see arrow) is consistent with the notion that transient CD69 expression (R3,R4) is obligatory for all thymocytes reaching R5. Fig. 1 *B* shows the expression of the coreceptors CD4 and CD8 in each of these popula-

Table 1. *Ratio of CD4 SP to CD8 SP Thymocytes in Selected Thymic Subpopulations*

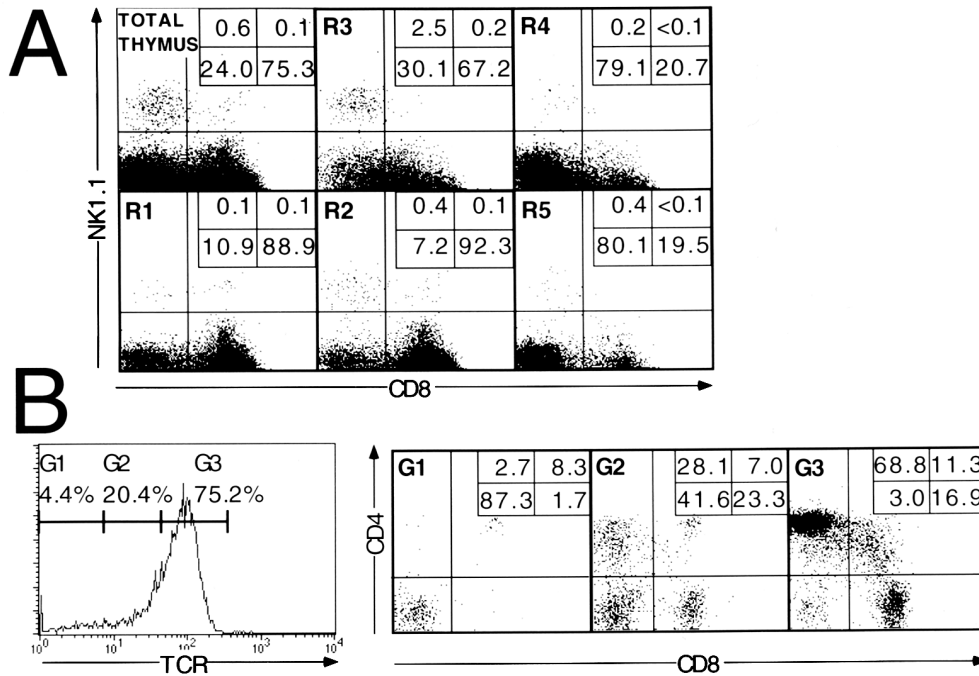|	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Total Thymus	3.1	2.3	3.0	3.6
R3, TCR^{int}CD69^{pos}*	14.2	9.8	10.0	14.9
R4, TCR^{hi}CD69^{pos}	6.3	6.7	7.5	6.9
R5, TCR^{hi}CD69^{neg}	1.7	1.2	1.4	1.7

*For settings of gates see original data in Fig. 1.

Figure 2. TCR^{int} CD69^{pos} DP and CD4 SP thymocytes are part of the mainstream αβ T cell lineage. (*A*) Total thymocytes were stained for TCR, CD69, CD8, and NK1.1 and analyzed by four-color flow cytometry. Gates R1–R5, defined by TCR and CD69, are the same as in Fig. 1. CD8 and NK1.1 expression in each gate is shown. (*B*) Development of CD4 SP and CD8 SP TCR^{hi} cells from sorted TCR^{int}CD69^{pos} thymocytes in RTOC. TCR^{int}CD69^{pos} thymocytes were purified by sorting; for analysis after sorting see Fig. 3 *A*. After 48 h of RTOC (for details see Materials and Methods), cells were analyzed for TCR, CD4 and CD8 expression. (*Left*) Histogram of TCR expression. (*Right*) CD4/CD8 distribution in gates defined by TCR levels.

tions. DP thymocytes account for most of the cells in R1 and R2 and decrease stepwise to less than 10% in R5. CD4 SP thymocytes are first observed in the TCR^{int}CD69^{pos} population (R3), whereas significant numbers of CD8 SP cells appear one stage later, i.e., in the TCR^{hi}CD69^{pos} population (R4). The ratios of CD4 SP to CD8 SP thymocytes decrease stepwise from more than 10 to ∼1.5 with ongoing maturation (Table 1). These data demonstrate that in vivo CD4 SP cells appear amongst cell populations which are at an earlier developmental stage than the populations containing CD8 SP cells.

TCR^{int}CD69^{pos} DP and CD4 SP Thymocytes Are Part of the Mainstream αβ T Cell Differentiation in the Thymus. A subpopulation of TCR^{int} CD4 SP cells with an activated phenotype and expressing the NK1.1 marker of natural killer cells has been described to occur in the thymus and other tissues (13, 14). This subset represents a lineage different from the major αβ and γδ T cell populations. To exclude the possibility that the early CD4 SP cells represent NK1.1^{pos} CD4 SP thymocytes, we analyzed the TCR^{int}CD69^{pos} CD8^{neg} population for NK1.1 expression. As shown in Fig. 2 *A*, the NK1.1 CD4 SP population is indeed enriched in R3, but accounts for a maximum of 10% of the total TCR^{int}CD69^{pos} CD4 SP population.

In addition, we tested the developmental potential of the TCR^{int}CD69^{pos} population in RTOC. TCR^{int}CD69^{pos} thymocytes were purified by cell sorting and reaggregated for 48 h with fetal stroma cells. As shown in Fig. 2 *B*, the majority of the thymocytes generated within 48 h were mature CD4 SP and CD8 SP cells with high TCR levels. The small proportion of TCR^{int} cells contained comparable amounts of CD4 SP and CD8 SP cells and thus also had further differentiated from the input TCR^{int} population.

Taken together, we conclude that the TCR^{int}CD69^{pos} thymocytes represent an intermediate developmental stage in mainstream differentiation and selection of αβ thymocytes.

Expression of Both Coreceptors in the TCR^{int}CD69^{pos} Population Requires Transcription and Translation. The failure to detect CD8 SP thymocytes in the most recently selected TCR^{int}CD69^{pos} thymocyte subset could be due a slow degradation of the CD4 protein, in contrast to a rapid disappearance of the CD8 protein from the cell surface after activation. As a consequence, the CD4 protein would still be present on the cell surface although the CD4 gene is turned off, thus letting CD8 SP thymocytes appear as DP cells for some time. To examine this possibility, sorted TCR^{int} CD69^{pos} thymocytes (Fig. 3 *A*) were treated with trypsin/ EDTA in order to strip the coreceptors from the cell surface (Fig. 3 *B*). After overnight culture, reexpression of CD4 and CD8 was analyzed. Cells that have terminated CD4 synthesis in the DP subset of the TCR^{int}CD69^{pos} thymocytes should become detectable as an increase in CD8 SP cells after trypsinization. As shown in Fig. 3 *C*, the number of CD8 SP cells after trypsinization and overnight incubation was only slightly greater than that in PBS-treated control thymocytes and did not exceed 1/20 of the number of CD4 SP cells regenerated after trypsinization. The level of CD4 reexpressed on trypsin-treated cells was not reduced compared to that on PBS-treated cells. Nevertheless, it was possible that the cells had sufficiently rich intracellular stores of CD4 protein to achieve complete redeposition of CD4 on the cell surface. To examine this possibility, overnight incubations after trypsin treatment were done in the presence of actinomycin D (ActD) or of cycloheximide (CHX) to inhibit transcription or translation, respectively. Whereas CHX completely blocked re-
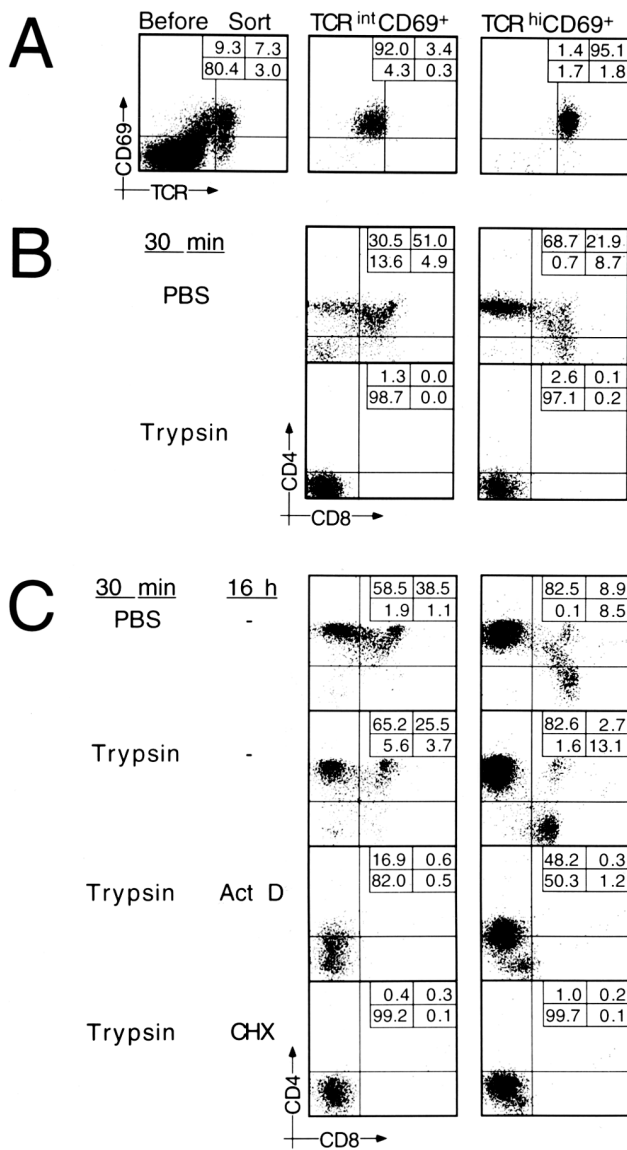
Figure 3. Proteolytic stripping and requirements for coreceptor reexpression on TCR^{int}CD69^{pos} and on TCR^{hi}CD69^{pos} thymocyte populations. (*A*) TCR^{int}CD69^{pos} and TCR^{hi}CD69^{pos} thymocytes were purified by cell sorting. (*B*) Purified cell populations were treated with PBS or trypsin, and immediately analysed for CD4/CD8 expression. (*C*) PBS-treated cells were incubated overnight in medium alone; trypsin-treated cells were incubated overnight in medium alone, or in the presence of ActD or CHX. Thereafter, cells were stained for CD4/CD8 expression. Percentages are given in the insets. Fluorescence intensities in *B* and *C* differ somewhat since analyses have been performed on different flow cytometers.

expression of both coreceptors, reappearance of low levels of CD4 on some of the cells was seen in the presence of ActD. The results suggest that coreceptor reexpression in this assay is completely dependent on translation, whereas some mRNA stores allow a limited reexpression of CD4. However, the data exclude a prolonged maintenance of CD4 surface expression after turnoff of CD4 biosynthesis.

For comparison we analyzed sorted TCR^{hi}CD69^{pos} thymocytes (Fig. 3 *A*), the first stage in which we see CD8 SP

cells. This population is particularly rich in cells of the so-called coreceptor skewed (CRS) phenotypes which are on their way to become SP cells, but still express low amounts of the inappropriate coreceptor. After trypsinization and overnight incubation both types of SP cells are fully regenerated (Fig. 3, *B* and *C*). The level of CD8 expression, however, is slightly decreased compared to the PBS control indicating that CD8 reexpression needs a longer time than CD4 reexpression. Cells with a CRS phenotype have largely disappeared. This decrease in CRS cells, seen to a lesser extent also after trypsinization of the TCR^{int}CD69^{pos} population, suggests that some of the CRS cells already have terminated biosynthesis of the inappropriate coreceptor. In this population, too, the reexpression of CD4 and CD8 is totally dependent on protein synthesis, but addition of ActD allows a low reexpression of CD4 on most of the cells (Fig. 3 *C*). This suggests that a low level of CD4 can indeed be transiently maintained by translation from long-lived stores of mRNA. However, this mechanism does not account for the delayed appearance of CD8 SP cells in thymic selection.

Discussion

In this study, we describe a population of recently selected thymocytes which still express the early activation marker CD69, but have so far not upregulated the TCR from intermediate to the high levels characteristic of mature SP thymocytes. Nevertheless, in addition to DP thymocytes, the TCR^{int}CD69^{pos} population contains a considerable fraction of CD4 SP cells, whereas CD8 SP cells can hardly be detected. These cells contain the precursors for mature TCR^{hi} CD4 SP and TCR^{hi} CD8 SP thymocytes, suggesting that they are part of mainstream $\alpha\beta$ T cell differentiation. NK1.1 thymocytes, some of which express CD4 together with intermediate TCR levels, only account for maximally 10% of the TCR^{int} CD4 SP cells. The data corroborate previous experiments suggesting that development of CD4 SP thymocytes precedes the development of CD8 SP thymocytes (6–12, 15).

The temporal asymmetry might be due to differences in protein turnover rates of CD4 and CD8, leading to a slower disappearance of CD4 than of CD8 from the cell surface following positive selection and turnoff of the respective genes. This question was investigated by proteolytic stripping of the coreceptors from recently selected thymocyte subpopulations, and observing their reexpression after incubation with and without inhibitors of transcription and translation. This type of assay has been successfully used by others to reveal cells which already have committed to either the CD4 or CD8 lineage (16). Our data show that the TCR^{int}CD69^{pos} population, although containing cells which have terminated biosynthesis of CD8, does not contain cells which have terminated biosynthesis of CD4. These data exclude a role for potentially trivial differences in protein turnover causing the asynchrony of the generation of CD4 SP and CD8 SP cells. Rather, the results suggest either distinct molecular matura-

tion kinetics or a sequential timing of selection signals in the CD4 and CD8 lineages. Furthermore, these data show that the CD8 committed cells present in the CD4^{pos}CD8^{int} CRS population (16, 17) are not present in significant amounts in the TCR^{int}CD69^{pos} population.

Temporally sequential selection would be conceivable if MHC class I and MHC class II molecules were presented in spatially separate compartments. Although no positive evidence exists suggesting this to be the case, it is so far not excluded that different cell types or anatomical niches mediate selection on class I and class II molecules. However, we doubt that experiments involving intrathymic injection or reaggregate organ cultures would uncover differences in selection based on subtle anatomical compartmentation. Since the temporal asymmetry in positive selection was primarily revealed by this type of experiment, a role of compartmentation seems unlikely. Rather, we think that selection signals delivered by interaction with MHC class I and MHC class II molecules initiate divergent molecular differentiation programs.

The principle ligands on thymocytes interacting with the two different MHC classes are the coreceptors CD4 and CD8. It is therefore tempting to speculate that signals transduced via the coreceptors themselves might control the differences in development of the two T cell lineages (18, 19). CD4 is known to associate with approximately five times more p56^{lck} than CD8 (20). It is therefore possible that distinct thresholds of signaling are required to complete positive selection of CD4 and CD8 cells, i.e., generation of CD4 SP cells requires higher signal strength than of CD8 cells. Recent data on selection in a MHC class II–restricted TCR transgenic mouse model support this type of mechanism. While normally generating mostly CD4 cells, CD8 SP thymocytes are generated when these TCR transgenic mice are made deficient of CD4 (21). These results suggest that signals delivered via the CD4 coreceptor override signals transduced by the CD8 coreceptor. Together with our data, a scenario emerges in which selection by MHC class II/CD4 interaction delivers strong signals and is definitive; selection by MHC class I/CD8 interaction delivers weak signals and is reversible as long as shutdown of CD4 has not taken place. The biological purpose of a reversible selection into the CD8 lineage could be to provide opportunities for rescue into the CD4 lineage for as many cells as possible.

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