Unraveling a Revealing Paradox: Why Major Histocompatibility Complex I–signaled Thymocytes "Paradoxically" Appear as $CD4^+8^{lo}$ Transitional Cells During Positive Selection of $CD8^+$ T Cells

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Abstract

The mechanism by which T cell receptor specificity determines the outcome of the CD4/CD8 lineage decision in the thymus is not known. An important clue is the fact that major histocompatibility complex (MHC)-I–signaled thymocytes paradoxically appear as $CD4^+8^{lo}$ transitional cells during their differentiation into $CD8^+$ T cells. Lineage commitment is generally thought to occur at the $CD4^+8^+$ (double positive) stage of differentiation and to result in silencing of the opposite coreceptor gene. From this perspective, the appearance of MHC-I–signaled thymocytes as $CD4^+8^{lo}$ cells would be due to effects on CD8 surface protein expression, not CD8 gene expression. But contrary to this perspective, this study demonstrates that MHC-I–signaled thymocytes appear as $CD4^+8^{lo}$ cells because of transient down-regulation of CD8 gene expression, not because of changes in CD8 surface protein expression or distribution. This study also demonstrates that initial cessation of CD8 gene expression in MHC-I–signaled thymocytes is not necessarily indicative of commitment to the $CD4^+$ T cell lineage, as such thymocytes retain the potential to differentiate into $CD8^+$ T cells. These results challenge classical concepts of lineage commitment but fulfill predictions of the kinetic signaling model.

Key words: lineage commitment • kinetic signaling • coreceptor reversal • positive selection

Introduction

Immature $CD4^+8^+$ (double positive [DP]*) thymocytes are signaled in the thymus to differentiate into mature single positive T cells by TCR engagement of intrathymic MHC–peptide complexes (1). TCR engagement of intrathymic peptide–MHC-II complexes results in differentiation of DP thymocytes into mature $CD4^+$ T cells whereas TCR engagement of intrathymic peptide–MHC-I complexes results in differentiation of DP thymocytes into mature $CD8^+$ T cells (2, 3). The mechanism by which the MHC class specificity of surface TCR complexes determines lineage choice remains an unresolved problem in developmental immunology. It is thought that lineage choice is a consequence of the coreceptor molecules that individual TCRs use to engage intrathymic ligands, as TCR engagement of peptide–MHC-II complexes generally requires coengagement of surface CD4 coreceptor molecules whereas TCR engagement of peptide–MHC-I complexes generally requires coengagement of surface CD8 coreceptor molecules (4, 5). However, the actual mechanism by which TCR and coreceptor coengagements influence lineage choice remains a matter of intense controversy.

Classical models of lineage commitment are of two general types: instructional and stochastic (6–10). Instructional models of lineage commitment propose that TCR plus CD4 coengagements on DP thymocytes terminate CD8 gene expression and dictate commitment to the $CD4^+$ T cell lineage by transducing signals that differ either qualitatively or quantitatively (in strength or duration) from signals transduced by TCR plus CD8 coengagements that terminate CD4 gene expression and dictate commitment to the $CD8^+$ T cell lineage (11–15). Stochastic models of lineage commitment propose that coengagement of TCR with either coreceptor molecule signals DP thymocytes to randomly terminate expression of one coreceptor gene,

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**Abbreviations used in this paper:* DP, double positive; hCD2, human CD2.

with only thymocytes still expressing matching TCR and coreceptor molecules responsive to subsequent survival signals (16, 17). In both models, lineage commitment is postulated to occur in DP thymocytes and result in silencing of the opposite coreceptor gene.

As a result, classical models of lineage commitment predict that DP thymocytes that have committed to the $CD4^+$ T cell lineage have terminated CD8 gene expression and therefore will lose CD8 surface protein expression and appear as $CD4^+8^{lo}$ transitional cells. Reciprocally, signaled DP thymocytes that have committed to the $CD8^+$ T cell lineage have terminated CD4 gene expression and will lose CD4 surface protein expression and appear as $CD4^{lo}8^+$ cells. However, experimental observations are discordant with these predictions as assessment of $CD4^+8^{lo}$ and $CD4^{lo}8^+$ transitional populations revealed that a significant proportion of CD8 lineage cells initially appear as $CD4^+8^{lo}$ transitional cells (18–22). The appearance of CD8-committed thymocytes as $CD4^+8^{lo}$ transitional cells is paradoxical on two levels, as: (a) CD8-committed thymocytes are thought to have terminated CD4 gene expression, yet these cells display high levels of CD4 proteins on their cell surface, and (b) CD8-committed thymocytes are thought to express CD8 coreceptor genes, yet these cells display low levels of CD8 proteins on their cell surface. Although the paradoxical loss of CD8 surface proteins on MHC-I–signaled thymocytes had not been predicted, it could be reconciled with classical lineage commitment models by hypothesizing that it was due to a direct effect of MHC-I signals on CD8 surface proteins that resulted in reduced CD8 expression on CD8-committed thymocytes (9, 22, 23). This classical view of $CD8^+$ T cell differentiation is depicted in Fig. 1 A.

In contrast, the kinetic signaling model of lineage commitment proposes that signaled DP thymocytes terminate CD8 gene expression and transcriptionally convert into $CD4^+8^-$ intermediate thymocytes that still retain the potential to differentiate into either $CD4^+$ or $CD8^+$ T cells (24–26). So kinetic signaling postulates that lineage commitment occurs after DP thymocytes have converted into $CD4^+8^-$ intermediate thymocytes. According to the kinetic signaling model, lineage direction is determined in $CD4^+8^-$ intermediate thymocytes simply by whether TCR-mediated positive selection signals persist or cease. If TCR signaling persists, intermediate $CD4^+8^-$ thymocytes differentiate into $CD4^+$ T cells, but if TCR signaling ceases, intermediate $CD4^+8^-$ thymocytes differentiate into $CD8^+$ T cells. As a result, a critical step in the differentiation of intermediate $CD4^+8^-$ thymocytes into $CD8^+$ T cells is the silencing of CD4 and reinitiation of CD8 gene expression, referred to as "coreceptor reversal." Consequently, the kinetic signaling model predicts that MHC-I–signaled thymocytes initially appear as $CD4^+8^{lo}$ cells because they initially terminate CD8 gene expression before undergoing coreceptor reversal to transcriptionally become $CD4^-8^+$ thymocytes. The kinetic signaling view of $CD8^+$ T cell differentiation in the thymus is depicted in Fig. 1 B.

Determining the basis for reduced CD8 surface protein expression on MHC-I–signaled thymocytes during $CD8^+$ T cell differentiation tests predictions made by different concepts of how lineage commitment occurs. The classical concept that lineage commitment occurs in DP thymocytes and results in irreversible silencing of the opposite coreceptor gene predicts that low CD8 surface protein expression on MHC-I–signaled $CD4^+8^{lo}$ thymocytes is due to direct effects on CD8 surface protein expression, not CD8 gene expression. In contrast, the kinetic signaling model predicts that low CD8 surface protein expression on MHC-I–signaled $CD4^+8^{lo}$ thymocytes is due to down-regulation of CD8 gene expression. Consequently, this study has attempted to determine the actual basis for reduced CD8 surface protein expression on MHC-I–signaled $CD4^+8^{lo}$ thymocytes. These results demonstrate that reduced CD8 surface expression on MHC-I–signaled $CD4^+8^{lo}$ thymocytes is not due to internalization nor slow reexpression of CD8 surface proteins, but is due to down-regulated CD8 gene expression. In addition, this study demonstrates that down-regulation of CD8 gene expression does not imply commitment to the CD4 lineage, as many intermediate $CD4^+8^-$ thymocytes retain the ability to differentiate into $CD8^+$ T cells. These results support the kinetic signaling model but challenge more classical concepts of lineage commitment.

Materials and Methods

Animals. Mice deficient in MHC-II ($MHC\text{-}II^o$; reference 27), CD8α ($CD8\alpha^o$; reference 5), or CD8β ($CD8\beta^o$; reference 28) were used at 8–12 wk of age and maintained in our own animal facility. $MHC\text{-}II^o$ mice were bred with $CD8\alpha^o$ mice to generate $II^oCD8\alpha^o$ mice, and were bred with $CD8\beta^o$ mice to generate $II^oCD8\beta^o$ mice.

CD8α transgenic mice constructed with human CD2 (hCD2)-based CD8α transgenes encoding either full-length CD8α or tailless CD8α′ molecules have been described (29, 30). Transgenic and endogenous CD8α proteins expressed different allelic forms of CD8α with transgenic CD8α proteins being CD8α.1 and endogenous CD8α proteins being CD8α.2. The CD8α transgenic mouse lines expressed transgenic and endogenous CD8α proteins at comparable levels. The CD8α.1 transgene was introduced by mating into $MHC\text{-}II^o$, $II^oCD8\alpha^o$, and $II^oCD8\beta^o$ mice to generate $MHC\text{-}II^o$ mice that either expressed both endogenous and transgenic CD8α proteins or expressed only transgenic CD8α proteins.

Line 30 transgenic mice expressing a transgene encoding hCD2 cDNA under the control of the CD4 locus control region (including the CD4 silencer element) were provided by D. Littman (Skirball Institute of Biomolecular Medicine, New York, NY; 31). Surface expression of hCD2 protein on thymocytes and T cells in Line 30 mice serves as a faithful reporter of CD4 promoter activity and strictly parallels CD4 gene expression (31). Line 30 mice were mated with $MHC\text{-}II^o$ mice to generate $MHC\text{-}II^o$ Line 30 mice.

Antibodies and Reagents. Fluorochrome-conjugated antibodies with the following specificities were used for direct immunofluorescence: pan CD8α (53-6.7; BD Biosciences), CD8α.1 (116-113.1; reference 32), CD8α.2 (2.43; reference 33), CD4 (GK1.5; BD Biosciences), TCRβ (H57-597; BD Biosciences), and hCD2 (G11; Caltag). Recombinant murine IL-7 (R&D Systems) was added to cultures where indicated.

Flow Cytometry and Electronic Cell Sorting. Cells were harvested, stained with fluorochrome-conjugated antibodies, and analyzed on a multi-laser FACSVantage™ SE (Becton Dickinson). Dead cells were excluded by forward light scatter gating and propidium iodide staining. Analysis was performed using software developed at the National Institutes of Health.

Pronase Treatment and the Coreceptor Reexpression Assay. Where indicated, thymocytes were pronase treated to remove preexisting surface CD4 and CD8 proteins (22). 5×10^6/ml cells were treated with 0.01% pronase (Calbiochem) for 15 min at 37°C and then cultured at 37°C overnight during which time they reexpressed the coreceptor molecules they were actively transcribing (22). For quantitative analysis of surface coreceptor reexpression, CD4 and CD8 fluorescence on thymocytes at various times after pronase stripping was quantitated in total fluorescence units and expressed as a percentage of CD4 and CD8 fluorescence on DP thymocytes after full coreceptor reexpression at 24 h, which was set equal to 100% and was comparable to CD4 and CD8 fluorescence on untreated DP thymocytes.

In Vitro Suspension Culture. Purified thymocyte subpopulations were placed in suspension cultures as previously described (24, 26). Where indicated, recombinant murine IL-7 was added at 6 ng/ml final concentration.

Results

To determine the mechanism by which CD8 surface protein expression is reduced on MHC-I–signaled thymocytes, we introduced a CD8α.1 transgene into MHC-II^o mice so that MHC-I–signaled thymocytes would express two different CD8α molecules, i.e., endogenously encoded CD8α.2 and transgenically encoded CD8α.1 molecules (29, 30). Endogenously encoded CD8α.2 proteins and transgene-encoded CD8α.1 proteins were expressed on the cell surface at similar levels and were identical except for the single amino acid change in the extracellular domain that is responsible for their allelism. However, the genes encoding endogenous and transgenic CD8α proteins were quite different from one another. The endogenous CD8α.2 gene utilizes CD8 promoter and CD8 regulatory elements, whereas the CD8α.1 transgene utilizes heterologous hCD2 promoter/enhancer elements (34). Thus, if MHC-I signaling initially reduces CD8 surface expression on DP thymocytes by directly affecting CD8 surface proteins, both endogenous CD8α.2 and transgenic CD8α.1 protein levels will be equally reduced as the two CD8 proteins are essentially identical. However, if MHC-I signaling initially reduces CD8 surface expression on DP thymocytes by targeting CD8 regulatory elements and down-regulating CD8 gene expression, surface expression of endogenously encoded CD8α.2 proteins will be reduced but surface expression of transgenically encoded CD8α.1 proteins will not be reduced.

Assessment of CD8 Protein Internalization as the Basis for Reduced CD8 Surface Protein Expression on MHC-I–signaled Thymocytes. Immature thymocytes that have been stimulated by intrathymic ligands to undergo positive selection up-regulate TCR surface expression to become TCR^{hi} cells. Gating on TCR^{hi} cells in MHC-II^o mice and assessing surface expression of endogenously encoded CD4 and CD8β molecules revealed two major populations of MHC-I–signaled thymocytes in both normal and CD8α transgenic mice: a transitional thymocyte population that is CD4$^+$ CD8β^{lo} and a mature population that is CD4$^-$ CD8β^{hi} (Fig. 2). Previous studies have documented that transitional CD4$^+$ CD8^{lo} cells are the progeny of MHC-I–signaled DP thymocytes that then differentiate into CD8$^+$ T cells (20–22). Importantly, it can be seen that surface expression of endogenous CD8α molecules paralleled that of surface CD8β molecules on both wild-type and CD8α transgenic mice, in that endogenous CD8α surface expression was low on CD8β^{lo} cells and high on CD8β^{hi} cells (Fig. 2). In sharp contrast, surface expression of transgenic CD8α molecules on CD8α transgenic thymocytes remained high on both CD8β^{lo} and CD8β^{hi} cells (Fig. 2). Thus, although intrathymic MHC-I signals in DP thymocytes reduced surface expression of endogenous CD8α molecules, MHC-I signals did not alter surface expression of transgenic CD8α molecules on the same cells. Because endogenous and transgenic CD8α proteins are essentially identical, these results indicate that intrathymic MHC-I signals do not reduce CD8α surface protein expression by inducing CD8α protein internalization.

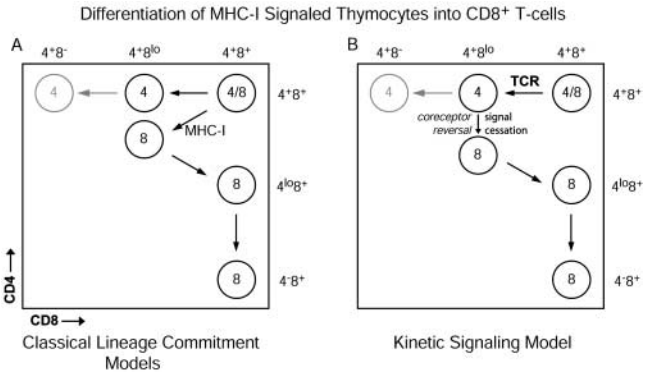


Figure 1. Alternative schematic representations of the mechanism by which MHC-I–signaled thymocytes differentiate into CD8$^+$ T cells. Differentiation of MHC-I–signaled thymocytes is schematized as a two color plot of surface CD4 versus CD8 expression of MHC-II^o thymocytes. Labels outside the plot identify thymocyte subpopulations by surface levels of CD4/CD8 protein expression. Labels inside the cells indicate coreceptor gene expression. Arrows indicate proposed precursor/progeny relationships according to either classical lineage commitment models (A) or the kinetic signaling model (B). Cells in light gray are absent in MHC-II^o thymi but present in normal thymi. (A) Classical lineage commitment models (e.g., instruction, stochastic/selection, strength of signal, duration of signal) are all based on the central paradigm that lineage commitment occurs in DP thymocytes and results in silencing of the opposite coreceptor gene. As a result, all classical models require that MHC-I–signaled thymocytes appear as CD4$^+$8^{lo} transitional cells because of changes in CD8 protein expression, not CD8 gene expression. (B) The kinetic signaling model proposes that TCR-signaled DP thymocytes initially terminate CD8 gene expression and appear as CD4$^+$8^{lo} transitional cells as a result of diminished or absent CD8 gene expression. In the kinetic signaling model, TCR disengagement of MHC-I ligands occurs in CD4$^+$8^{lo} transitional cells because of diminished surface CD8 coreceptor levels and results in cessation of TCR signaling, reversal of coreceptor gene expression, and terminal differentiation into CD8$^+$ T cells.

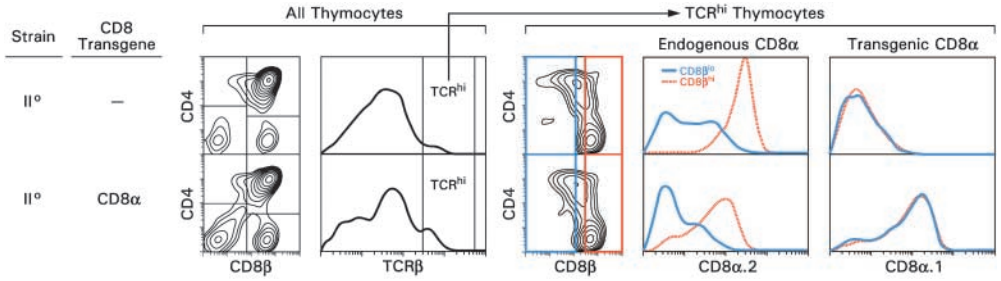


Figure 2. Differential expression of endogenous and transgenic CD8α expression on thymocytes signaled in vivo by intrathymic MHC-I complexes. Thymocytes from MHC-II^{o} mice (either with or without the CD8α transgene) were surface stained for TCRβ, CD4, CD8β, and either CD8α.2 (endogenous CD8α) or CD8α.1 (transgenic CD8α) expression and analyzed by four color flow cytometry. Shown on whole thymus populations from each mouse are CD4 versus CD8β and TCRβ expression (left panels). Because intrathymically signaled thymocytes up-regulate surface TCR expression and become TCR^{hi} cells, we gated on TCR^{hi} thymocytes to determine surface coreceptor expression on in vivo–signaled cells (right panels). CD4 versus CD8β expression on TCR^{hi} thymocytes is shown, with CD8β^{lo} cells identified by the blue rectangular gate and CD8β^{hi} identified by the red rectangular gate. CD8α staining on CD8β^{lo} (blue line) and CD8β^{hi} (red line) TCR^{hi} thymocytes was compared for surface expression of endogenous CD8α.2 and transgenic CD8α.1 molecules on the identical cells. Surface expression of endogenous CD8α.2 molecules was found to parallel that of CD8β, in that CD8α.2 expression was low on CD8β^{lo} cells and high on CD8β^{hi} TCR^{hi} cells. In contrast, surface expression of transgenic CD8α.1 molecules did not parallel that of CD8β, in that CD8α.1 expression was identical on CD8β^{lo} and CD8β^{hi} TCR^{hi} thymocytes.

Although transgenic CD8α molecules are essentially identical to full-length endogenous CD8α molecules, thymocytes also express a splice variant of endogenous CD8α (referred to as CD8α′) lacking a cytosolic tail (35). Consequently, we considered the unlikely possibility that intrathymic MHC-I signals might have reduced surface expression of endogenous, but not transgenic, CD8α molecules on CD8β^{lo} cells by selectively internalizing CD8 complexes containing a CD8α′ chain. We introduced either the full-length CD8α or tailless CD8α′ transgene into II^{o}CD8α^{o} mice that lack endogenous CD8α expression so that all surface CD8αβ complexes would contain only CD8α transgenic molecules (Fig. 3). Gating on TCR^{hi} cells and assessing surface expression of endogenously encoded CD4 and CD8β molecules revealed the same two major populations of MHC-I–signaled thymocytes in both CD8α and CD8α′ transgenic mice: a transitional thymocyte population that was CD4^{+}8β^{lo} and a mature population that was CD4^{-}8β^{hi}. Importantly, surface expression of either full-length or tailless CD8α transgenic molecules was only slightly reduced on CD8β^{lo} thymocytes relative to CD8β^{hi} thymocytes (Fig. 3). These results exclude the unlikely possibility that intrathymic MHC-I signals reduce surface CD8 protein expression by selectively internalizing CD8α′ complexes.

Because surface CD8αβ heterodimers bind to MHC-I molecules with greater avidity than do CD8αα homodimers (30), it was conceivable that intrathymic MHC-I engagements might result in selective internalization of CD8αβ complexes but not CD8αα complexes. Consequently, we considered that intrathymic MHC-I signals might have failed to reduce surface expression of transgenic CD8α molecules because transgenic CD8α molecules were expressed as CD8αα homodimers whereas endogenous CD8α molecules were expressed as CD8αβ heterodimers. To assess this possibility, we introduced the CD8α transgene into II^{o}CD8β^{o} mice so that the only CD8 complexes thymocytes could express would be CD8αα complexes composed of endogenous and/or transgenic CD8α molecules (Fig. 4). Gating on TCR^{hi} cells and assessing surface expression of endogenously encoded CD4 and CD8α.2 molecules again revealed two major populations of MHC-

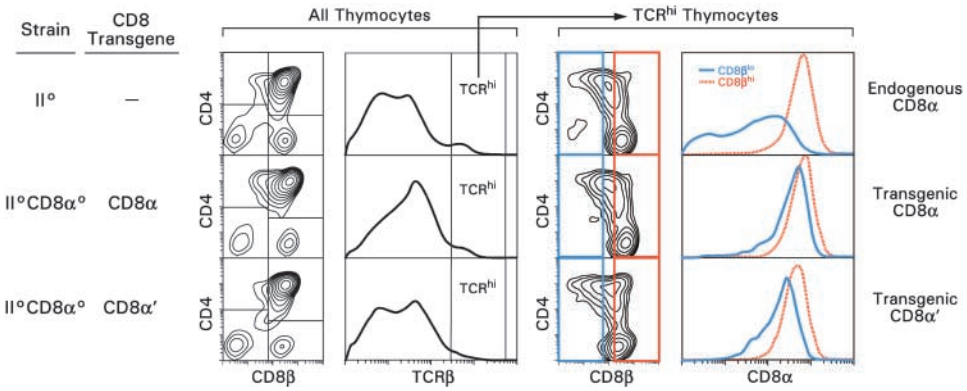


Figure 3. Effect of intrathymic MHC-I signals on surface expression of full-length CD8α and tailless CD8α′ transgenic molecules. Thymocytes were obtained from MHC-II^{o} mice that expressed only endogenous CD8 molecules (both CD8α and CD8α′; top row), or from MHC-II^{o} CD8α^{o} mice that either expressed only full-length CD8α (middle row) or tailless CD8α′ (bottom row) transgenic molecules. Thymocytes were subjected to the same four color flow cytometric analysis as in Fig. 2, with the exception that both endogenous and transgenic CD8α molecules were stained with a pan anti-CD8α mAb. In contrast to endogenous CD8α molecules that were expressed at significantly lower levels on CD8β^{lo} (blue line) than CD8β^{hi} (red line) TCR^{hi} thymocytes, transgenic CD8α molecules were expressed at similar levels on CD8β^{lo} and CD8β^{hi} TCR^{hi} thymocytes regardless of whether the transgene encoded full-length CD8α or tailless CD8α′ molecules.

I–signaled thymocytes: a transitional thymocyte population that was $CD4^+$ $CD8\alpha.2^{lo}$ and a mature population that was $CD4^-$ $CD8\alpha.2^{hi}$ (Fig. 4). Even though all surface CD8 complexes on these thymocytes could only be CD8αα homodimers, surface expression of endogenous CD8α.2 molecules was obviously reduced on $CD8\alpha.2^{lo}$ thymocytes, whereas surface expression of transgenic CD8α.1 molecules was not reduced on the very same $CD8\alpha.2^{lo}$ cells (Fig. 4). The detection of TCR^{hi} transitional $CD4^+$ $CD8\alpha.2^{lo}$ thymocytes in $II^oCD8\beta^o$ mice directly excludes the possibility that intrathymic MHC-I signals fail to reduce surface expression of CD8αα homodimers.

These results demonstrate that MHC-I–signaled $CD4^+8^{lo}$ thymocytes display reduced surface expression of all endogenously encoded CD8 proteins (CD8α, CD8α′, and CD8β), but not any transgene-encoded CD8 proteins (CD8α, CD8α′). Because endogenously encoded and transgene-encoded CD8α proteins are essentially identical, these results demonstrate that CD8 protein internalization cannot be the mechanism by which intrathymic MHC-I signals reduce CD8 surface protein expression and convert positively selected DP thymocytes into transitional $CD4^+8^{lo}$ cells.

Assessment of CD8 Protein Externalization as the Basis for Reduced CD8 Surface Protein Expression on MHC-I–signaled Thymocytes. Having excluded CD8 protein internalization as the basis for reduced CD8 surface protein expression on MHC-I–signaled thymocytes, we next considered whether CD8 protein externalization might be the explanation. It has been proposed that TCR-signaled DP thymocytes undergo a complex set of changes in coreceptor surface protein expression that occur independently of, and before, changes in coreceptor gene expression (9, 23). In this proposal, TCR-signaled DP thymocytes initially remove both CD4 and CD8 proteins from the cell surface to phenotypically convert into $CD4^{lo}8^{lo}$ thymocytes that then phenotypically become $CD4^+8^{lo}$ cells because CD4 proteins are more rapidly reexpressed on the cell surface than CD8 proteins. Thus, transgenic CD8α proteins might also be more rapidly reexpressed on the cell surface than endogenous CD8α proteins, explaining why $CD4^+8^{lo}$ cells expressed low surface levels of endogenous CD8α proteins but high surface levels of transgenic CD8α proteins (Figs. 2–4). Consequently, we compared relative reexpression rates of transgenic and endogenous CD8 surface proteins on thymocytes after surface coreceptor proteins had been stripped away by treatment with the extracellular protease, pronase. We did this in two different ways. We used allele-specific anti-CD8α mAbs to determine reexpression rates of endogenous CD8α.2 and transgenic CD8α.1 proteins on DP thymocytes expressing both CD8α proteins (Fig. 5 A), and we used a pan-CD8α mAb to determine reexpression rates of endogenous CD8α.2 and transgenic CD8α.1 proteins on DP thymocytes expressing only one or the other CD8α protein (Fig. 5 B). In both experimental situations, CD4 proteins were reexpressed on the cell surface at a faster rate than CD8 proteins and could be detected on the cell surface first (Fig. 5, A and B), as originally described (23). However, endogenous and transgenic CD8α proteins were reexpressed on the cell surface at identical rates, regardless of how this experiment was performed (Fig. 5, A and B). Consequently, for differential reexpression rates of coreceptor proteins to be the explanation for MHC-I–signaled $CD4^+8^{lo}$ thymocytes, $CD4^+8^{lo}$ thymocytes would have to be low for expression of both endogenous and transgenic CD8α proteins as their reexpression rates are identical. But, despite identical reexpression rates, MHC-I–signaled $CD4^+8^{lo}$ thymocytes only exhibited low surface levels of endogenously encoded CD8α proteins but exhibited high surface levels of transgenically encoded CD8α proteins (Figs. 2–4). Thus, the phenotypic appearance of MHC-I–signaled thymocytes as $CD4^+8^{lo}$ cells is not due to differential rates of coreceptor protein externalization.

We conclude that the appearance of MHC-I–signaled thymocytes as $CD4^+8^{lo}$ cells is neither due to rapid internalization nor slow externalization of CD8 surface proteins. Rather, the difference in expression of endogenous and transgenic CD8α molecules on MHC-I–signaled $CD4^+8^{lo}$ thymocytes must reflect the impact of TCR signals on endogenous CD8 regulatory elements that are not present in the hCD2-based transgene and result in the selective down-regulation of endogenous CD8 gene expression.

Initial Cessation of CD8 Gene Expression Does Not Imply Lineage Commitment. If MHC-I signals down-regulate CD8 gene expression in positively selected DP thymocytes

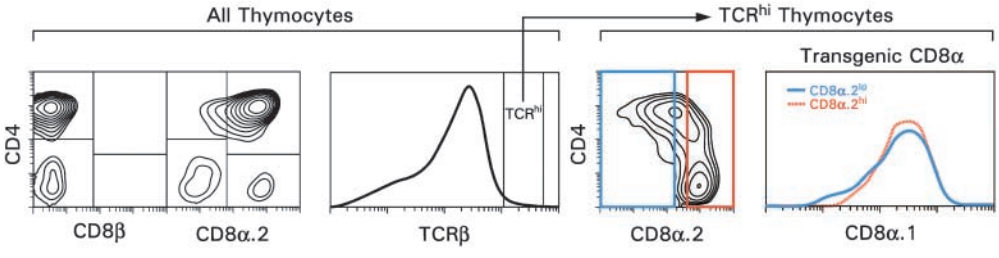
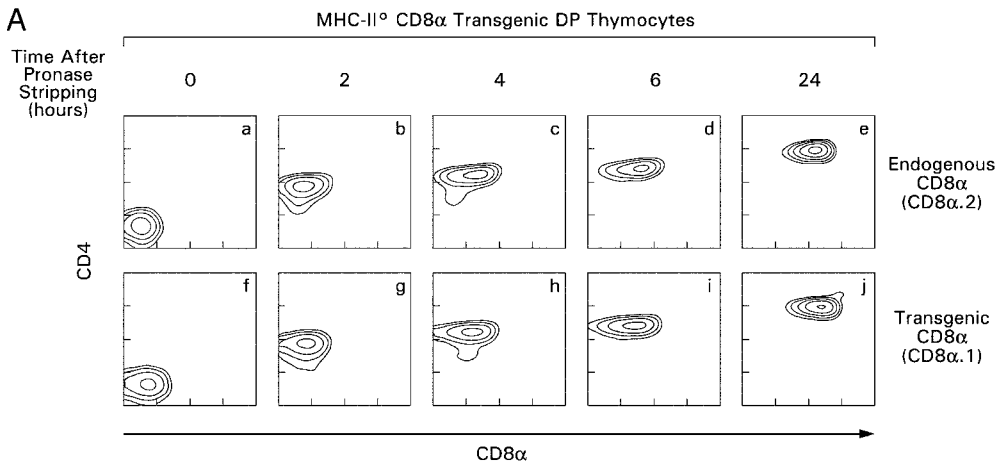


Figure 4. Effect of intrathymic MHC-I signals on surface expression of CD8αα homodimeric complexes. Thymocytes were obtained from MHC-II^o $CD8\beta^o$ mice that expressed both endogenous CD8α.2 and transgenic CD8α.1 molecules as CD8αα complexes. Thymocytes were surface stained for TCRβ, CD4, CD8β, and either CD8α.2 (endogenous CD8α) or CD8α.1 (transgenic CD8α) expression and analyzed by four color flow cytometry. Shown on whole thymus populations are CD4 versus CD8β expression, CD4 versus CD8α.2 expression, and TCRβ expression (left panels). CD4 versus CD8α.2 expression on gated TCR^{hi} thymocytes is shown and identifies $CD8\alpha.2^{lo}$ cells (mostly $CD4^+$ cells, blue rectangular gate) and CD8 $\alpha.2^{hi}$ cells (mostly $CD4^-$ cells, red rectangular gate). CD8α.1 surface expression was found to be similar on $CD8\alpha.2^{lo}$ (blue line) and $CD8\alpha.2^{hi}$ (red line) TCR^{hi} thymocytes (right panel).



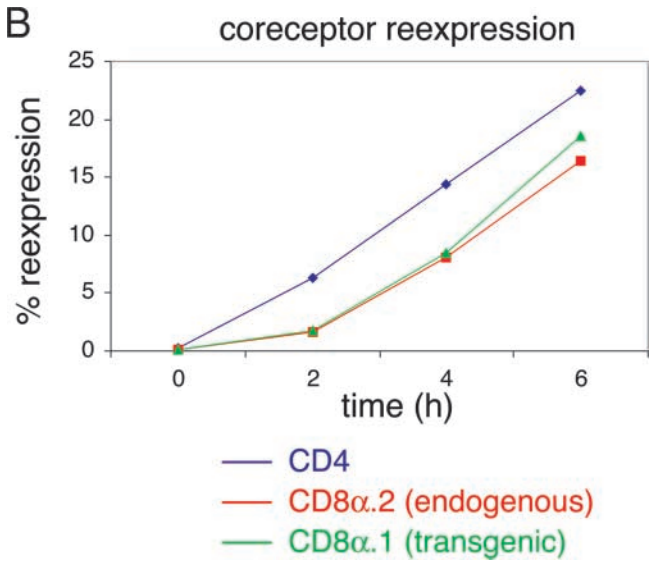


Figure 5. Reexpression rates of endogenous and transgenic coreceptor proteins. (A) Thymocytes from MHC-II° mice that carried the CD8α transgene and expressed both endogenous CD8α.2 and transgenic CD8α.1 molecules were stripped of all surface coreceptor proteins by treatment with extracellular pronase. The stripped thymocytes were cultured in single cell suspensions at 37°C for varying times, and assessed for reexpression of CD4, CD8α.2, and CD8α.1 by staining with anti-CD4 and allele-specific anti-CD8 mAbs. Displayed are histograms of CD4 versus CD8α.2 and CD4 versus CD8α.1, reexpression at different times after pronase stripping. Although CD4 reexpression occurred more quickly than CD8 reexpression, the kinetics of reexpression of endogenous CD8α.2 and transgenic CD8α.1 molecules were identical. (B) Thymocytes were obtained from MHC-II° mice expressing only endogenous CD8α.2 molecules, and from MHC-II° CD8α° mice that contained the CD8α transgene and therefore expressed only transgenic CD8α.1 molecules. Thymocytes were stripped of surface coreceptor proteins by treatment with extracellular pronase, cultured in single cell suspensions at 37°C for varying times, and assessed for reexpression of CD4 and CD8α by staining with anti-CD4 and a pan anti-CD8 mAb that recognizes both CD8α.1 and CD8α.2 isoforms. Line graphs show quantitative reexpression of CD4 and CD8 surface proteins relative to their maximum reexpression at 24 h, which was set equal to 100%. The kinetics of CD4 reexpression were identical on thymocytes from MHC-II° and CD8α transgenic MHC-II° CD8α° mice, but are displayed only for the former. Quantitation of surface reexpression versus time of CD4 (blue line), endogenous CD8α.2 (red line), and transgenic CD8α.1 (green line) are displayed.

thereby causing them to become transitional $CD4^+8^{lo}$ cells before their terminal differentiation into $CD8^+$ T cells, MHC-I–signaled $CD4^+8^{lo}$ thymocyte populations should contain two distinct subpopulations: one subpopulation consisting of signaled (i.e., TCR^{hi}) thymocytes that are transcriptionally $CD4^+8^-$, and the other subpopulation consisting of signaled TCR^{hi} cells that are the immediate progeny of $CD4^+8^-$ cells that have just undergone coreceptor reversal and become transcriptionally $CD4^-8^+$. To experimentally identify such subpopulations among MHC-I–signaled $CD4^+8^{lo}$ thymocytes, we subjected sorted $CD4^+8^{lo}$ thymocytes to the coreceptor reexpression assay in which they were treated with extracellular pronase to remove preexisting CD4 and CD8 surface coreceptor proteins and cultured overnight to allow surface reexpression of the coreceptor molecules actively being transcribed (Fig. 6; reference 22). Note that we have previously documented that the coreceptor reexpression assay reveals the coreceptor genes that individual thymocytes are actively transcribing and the coreceptor mRNAs that they contain (22, 24). Applying the coreceptor reexpression assay, we found that $CD4^+8^-$ and $CD4^-8^+$ TCR^{hi} subpopulations were both present among electronically sorted $CD4^+8^{lo}$ thymocytes from MHC-II° mice (Fig. 6, populations a and c). Also unavoidably included in the $CD4^+8^{lo}$ sorting gate were unsignaled (i.e., TCR^{lo}) DP thymocytes that reexpressed both CD4 and CD8 (Fig. 6, population b), and unsignaled TCR^{lo} DP thymocytes that were presumably in the early stages of apoptosis and so reexpressed neither CD4 nor CD8 (Fig. 6). Importantly, both of these latter subpopulations were TCR^{lo}, indicating that they had not been signaled in vivo (Fig. 6). Thus, this experiment confirms that MHC-I–signaled TCR^{hi} thymocytes that phenotypically appear as $CD4^+8^{lo}$ cells actually consist of two TCR^{hi} subpopulations that, as revealed by the coreceptor reexpression assay, are $CD4^+8^-$ and $CD4^-8^+$.

Consequently, we asked if in vivo MHC-I–signaled TCR^{hi} $CD4^+8^{lo}$ thymocytes that reexpressed only CD4 coreceptor proteins and therefore were $CD4^+8^-$ for coreceptor gene expression still retained the potential to differ-

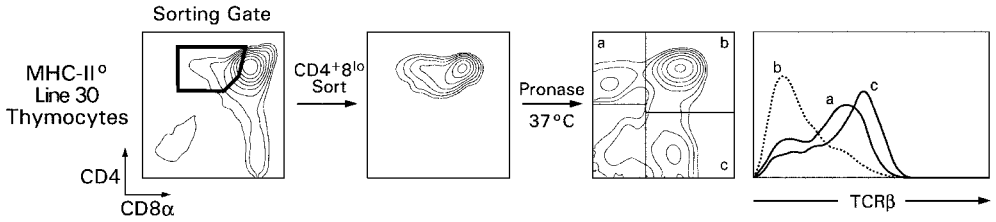


Figure 6. Analysis of MHC-I–signaled (TCR^{hi}) $CD4^+8^{lo}$ transitional thymocytes by the coreceptor reexpression assay. Thymocytes obtained from MHC-II° Line 30 mice were electronically sorted to obtain cells enriched in $CD4^+8^{lo}$ transitional thymocytes. Line 30 mice contain a reporter transgene in which surface expression of the hCD2 reporter protein reflects CD4 promoter activity (reference 31). The sorted thymocytes were pronase stripped, cultured overnight in 37°C suspension cultures, and assessed by multicolor flow cytometry for expression of CD4, CD8α, and TCRβ. $CD4^+8^{lo}$ transitional thymocytes consist primarily of two TCR^{hi} subpopulations as determined by the coreceptor reexpression assay: a $CD4^+8^-$ subpopulation (a) and a $CD4^-8^+$ subpopulation (c). Sorted $CD4^+8^{lo}$ thymocytes that either reexpressed both CD4 and CD8 (b), or neither CD4 nor CD8, were TCR^{lo} and therefore considered to have not been signaled in vivo. Note that previous analyses have documented that CD4 and CD8 protein reexpression in the coreceptor reexpression assay parallels CD4 and CD8 mRNA expression as assessed by RT-PCR (references 24 and 26).

entiate into $CD4^-8^+$ cells. To address this question, we used MHC-II° mice containing a reporter transgene (referred to as Line 30; reference 31) that is composed of hCD2 cDNA under the control of CD4 enhancer and silencer elements and therefore faithfully reports in vivo CD4 transcription by surface expression of hCD2 reporter protein. We obtained in vivo–signaled $CD4^+8^{lo}$ thymocytes from such MHC-II° Line 30 mice by electronic cell sorting (Fig. 7, a and b), identified the subpopulation that was transcriptionally $CD4^+8^-$ in the coreceptor reexpression assay (Fig. 7, d), and purified cells that were $CD4^+8^-$ $hCD2^+$ by further electronic sorting (Fig. 7, c). The purified cells were then placed into short-term cultures with IL-7, a cytokine present in the normal thymus, to maintain cell viability (24, 26). Unlike sorted $CD4^+8^+$ cells that remained DP in culture (Fig. 7, g and i), most $CD4^+8^-$ $hCD2^+$ cells underwent a dramatic change, terminating CD4 gene transcription (as indicated by absent CD4 and absent hCD2 reexpression) and reinitiating CD8 gene expression (as revealed by CD8 reexpression; Fig. 7, f and h). Thus, most in vivo MHC-I–signaled $CD4^+8^-$ thymocytes underwent coreceptor reversal in vitro, converting into $CD4^-8^+$ cells. A minority of $CD4^+8^-$ $hCD2^+$ cells did not change coreceptor gene expression in culture (Fig. 7, f and h).

We conclude that intrathymic MHC-I–signaled DP thymocytes phenotypically convert into $CD4^+8^{lo}$ cells because they have transiently terminated CD8 gene expression to become $CD4^+8^-$ cells. We also conclude that many such $CD4^+8^-$ cells remain lineage uncommitted as they retain the potential to reverse coreceptor gene expression and to differentiate into $CD4^-8^+$ cells.

Discussion

Most models of lineage commitment incorporate the concept that lineage commitment occurs in DP thymocytes and results in permanent silencing of the opposite coreceptor gene (9, 10). From this perspective the paradoxical appearance of MHC-I–signaled DP thymocytes as $CD4^+8^{lo}$ intermediates would be due to removal or redistribution of CD8 surface proteins, and not due to down-regulation of CD8 gene expression. However, this study documents that removal or redistribution of CD8 surface proteins is not the basis for conversion of MHC-I–signaled DP thymocytes into $CD4^+8^{lo}$ cells, but rather that it is due to down-regulation of CD8 gene expression. This study also demonstrates that MHC-I–signaled thymocytes that have down-regulated CD8 gene expression to become $CD4^+8^-$ cells still retain the potential to differentiate into $CD8^+$ T cells. Thus, initial cessation of CD8 gene expression does not imply commitment to the $CD4^+$ T cell lineage, as it occurs in MHC-I–signaled thymocytes during differentiation into $CD8^+$ T cells.

That many MHC-I–signaled DP thymocytes phenotypically convert into $CD4^+8^{lo}$ cells during differentiation into CD8SP T cells was a surprising observation made independently by two different laboratories (21, 22) that has since been confirmed by others (18, 19, 23, 36). Importantly, MHC-I–signaled $CD4^+8^{lo}$ thymocytes are not dead-end cells, but are cells on their way to differentiating into CD8SP T cells (18, 21, 22). Nevertheless, the molecular basis for this observation has been uncertain. Two explanations have been proposed that can reconcile the appearance of MHC-I–signaled $CD4^+8^{lo}$ thymocytes with the concept that lineage commitment occurs in DP thymocytes and results in permanent silencing of the opposite coreceptor gene. The first explanation posits that MHC-I–specific TCR interactions signal DP thymocytes to internalize surface CD8 coreceptor proteins even as they induce DP thymocytes to terminate CD4 gene expression (22). The second explanation is more complex and posits that MHC-I–specific TCR interactions signal DP thymocytes to remove both CD4 and CD8 coreceptor proteins from the cell surface that are then reexpressed at different rates, with the effect that MHC-I–signaled DP thymocytes transiently appear as $CD4^+8^{lo}$ cells though they continue to actively express both coreceptor genes (9, 23). Both explanations have now been excluded by this study.

This study used an hCD2-based CD8α.1 transgene that is expressed on thymocytes at similar levels to that of endogenous CD8α.2 proteins. Endogenous and transgenic CD8α proteins are essentially identical, but the genes encoding them are controlled by entirely different regulatory elements, as expression of endogenous CD8α genes is controlled by endogenous CD8 promoter/enhancer elements

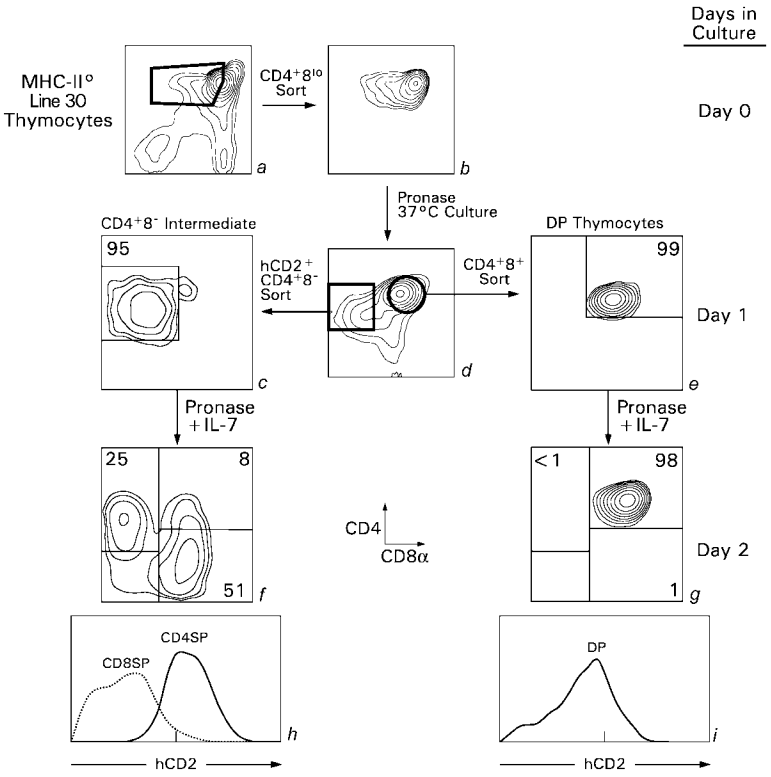


Figure 7. MHC-I–signaled $CD4^+8^{lo}$ thymocytes that have ceased CD8 gene expression retain the ability to differentiate into $CD8^+$ T cells. Thymocytes from MHC-II° Line 30 mice (a) were electronically sorted to obtain cells enriched in $CD4^+8^{lo}$ transitional thymocytes (b), which were then pronase stripped, cultured overnight, and assessed by multicolor flow cytometry for reexpression of CD4, CD8α, and the hCD2 reporter protein (d). To determine if coreceptor gene expression was fixed, purified $hCD2^+$ $CD4^+8^-$ cells (c) and $CD4^+8^+$ cells (e) were cultured in IL-7 and assessed for coreceptor reexpression (f and g). Cultured cells were also assessed for reexpression of the hCD2 protein that serves as a reporter of CD4 promoter activity (h and i). Most $CD4^+8^{lo}$ transitional thymocytes that had ceased CD8 gene expression and therefore were $hCD2^+$ $CD4^+8^-$ at the beginning of IL-7 culture (c), underwent coreceptor reversal and converted into $hCD2^-$ $CD4^-CD8^+$ cells (f and h). Thymocytes that had not been signaled in vivo and therefore expressed both CD4 and CD8 coreceptor genes did not alter coreceptor gene expression in culture and remained $hCD2^{lo}$ $CD4^+8^+$ (g and i).

(37–42) whereas expression of the CD8α transgene is controlled by heterologous hCD2 promoter/enhancer elements (29, 34). This study found that MHC-I–signaled $CD4^+8^{lo}$ thymocytes expressed low surface levels of endogenously encoded CD8α proteins but expressed high surface levels of transgenically encoded CD8α proteins, an observation that is not consistent with a direct effect of MHC-I signals on CD8α surface protein expression. Nevertheless, to document that internalization of CD8 surface complexes was not the mechanism by which MHC-I–signaled thymocytes appeared in vivo as $CD4^+8^{lo}$ cells, we evaluated and excluded the possibility that MHC-I signals internalized only specific subsets of CD8 surface protein complexes, such as those containing only tailless CD8α′ proteins, those consisting only of CD8αβ heterodimeric complexes, or only of CD8αα homodimeric complexes. And to document that slow externalization of CD8 protein complexes was not the mechanism by which MHC-I–signaled thymocytes appeared in vivo as $CD4^+8^{lo}$ cells, we evaluated and excluded the possibility that transgenic CD8α proteins, like CD4 proteins, might be reexpressed on the cell surface at a faster rate than endogenous CD8α proteins (23). Consequently, regardless of whether MHC-I signals stimulate the removal and/or reexpression of surface coreceptor proteins, this study demonstrates that such events are not the mechanism by which MHC-I–signaled thymocytes convert in vivo into $CD4^+8^{lo}$ thymocytes. Rather, the appearance of MHC-I–signaled thymocytes as $CD4^+8^{lo}$ cells must result from transient down-regulation of endogenous CD8 gene expression.

Supporting this conclusion, we found that in vivo MHC-I–signaled $CD4^+8^{lo}$ thymocytes consisted of two subpopulations of TCR^{hi} cells with different patterns of coreceptor gene expression: one subpopulation that had indeed down-regulated CD8 gene expression to become $CD4^+8^-$, and the other subpopulation whose coreceptor gene expression was $CD4^-8^+$. That the transitional $CD4^+8^{lo}$ population contained TCR^{hi} thymocytes with a coreceptor gene expression pattern of $CD4^+8^-$ was concordant with our conclusion that MHC-I–signaled thymocytes phenotypically appeared as $CD4^+8^{lo}$ cells because of down-regulated CD8 gene expression. Indeed, this study demonstrates that despite having ceased CD8 gene expression, such $CD4^+8^-$ cells could undergo coreceptor reversal into $CD4^-8^+$ cells in the presence of IL-7, a cytokine that is present within the normal thymus (43). As a result, TCR^{hi} cells that are transcriptionally $CD4^-8^+$ and in the process of differentiating into mature $CD8^+$ T cells are also present within the transitional $CD4^+8^{lo}$ thymocyte population because they are the immediate progeny of cells that were transcriptionally $CD4^+8^-$ before undergoing coreceptor reversal. Thus, cessation of CD8 gene expression in in vivo–signaled thymocytes does not necessarily imply CD4 lineage commitment, as such cells may undergo coreceptor reversal and differentiate into $CD8^+$ T cells (25).

It seems to us that the present results cannot be easily reconciled with the concept that lineage commitment occurs in DP thymocytes and results in permanent silencing of the opposite coreceptor gene. Cessation of coreceptor

gene expression was originally equated with commitment to the opposite coreceptor lineage (6, 16). For example, the original instructional model postulated that MHC-specific TCR plus coreceptor engagements dictated which coreceptor gene was silenced, whereas stochastic models postulated that the selection of which coreceptor gene to silence occurred randomly. Updated versions of the original instruction model no longer equate cessation of coreceptor gene expression with lineage commitment, but postulate that the strength or duration of the TCR signal in DP thymocytes commits the cell to one coreceptor lineage and that lineage choice is revealed by its subsequent silencing of the opposite coreceptor gene (11, 14, 15, 44). Importantly, cessation of coreceptor gene expression is still considered to reflect an individual cell's "commitment" to the opposite coreceptor lineage. In contrast, the present results are consistent with the kinetic signaling perspective that intermediate $CD4^+8^-$ thymocytes are lineage-uncommitted cells in which lineage commitment then occurs. In the kinetic signaling model, transient cessation of CD8 gene expression occurs during differentiation of MHC-I–signaled DP thymocytes and results in cessation of CD8-dependent MHC-I–specific signaling which, in turn, promotes coreceptor reversal and differentiation into CD8SP T cells (24, 25). However, not all MHC-I–signaled thymocytes go through a stage where they appear as $CD4^+8^{lo}$ cells. For example, DP thymocytes that express TCR with low apparent affinity for intrathymic MHC-I ligands appear to differentiate directly into CD8SP T cells without ever appearing as $CD4^+8^{lo}$ cells (36, 45). It is possible that such MHC-I–signaled cells do transiently down-regulate CD8 gene expression as proposed by the kinetic signaling model, but their TCR signals are of such short duration as a result of low ligand affinity that these cells undergo coreceptor reversal and reinitiate CD8 gene expression before surface CD8 protein levels can detectably decline (25). Whether or not signals transduced by low affinity MHC-I–specific TCR in fact induce transient down-regulation of CD8 gene expression before undergoing lineage commitment remains to be determined.

This study demonstrates that the appearance of MHC-I–signaled $CD4^+8^{lo}$ thymocytes is due to down-regulation of CD8 gene expression rather than to effects on CD8 surface proteins, but it is possible that MHC-I–specific TCR signals may affect CD8 mRNA stability as well as CD8 transcription. Our present observation that MHC-I–specific TCR signals cause DP thymocytes to discontinue expression of all endogenously encoded CD8 genes (CD8α and CD8β) without affecting expression of CD8 transgenes indicates that TCR signals target regulatory elements present within the endogenous CD8 gene locus that are absent from the hCD2-based transgene. Indeed, in vitro observations from this laboratory have previously demonstrated that antibody-mediated TCR engagement of purified DP thymocytes selectively terminates endogenous CD8 gene transcription and modestly destabilizes both CD4 and CD8 coreceptor mRNAs (46, 47). We think that similar events are likely occurring in in vivo–signaled DP thymocytes.

Although our understanding of CD4 and CD8 gene transcription is still far from complete, current knowledge of how CD8 and CD4 genes are transcriptionally regulated provides some clues as to how TCR signaling in DP thymocytes might selectively result in transient cessation of CD8 gene expression and conversion of signaled DP thymocytes into $CD4^+8^-$ cells. To oversimplify the situation in developing thymocytes, lineage-specific expression of CD8 and CD4 coreceptor genes appears to be regulated by fundamentally opposite mechanisms, as CD8 gene expression is specifically up-regulated by activation of stage-specific CD8 enhancer elements (37–42) and CD4 gene expression is specifically down-regulated by activation of a CD4 silencer element (31, 48, 49). As a result, the "basal" or "default" state of coreceptor gene expression in developing thymocytes might be considered to be $CD4^+8^-$, as this represents a transcriptional state in which neither CD8 enhancer elements nor CD4 silencer elements are activated. However, enhancer usage within the CD8 gene locus is known to shift during differentiation of immature DP thymocytes into mature $CD8^+$ T cells, as "immature" CD8 enhancer elements drive CD8 gene expression at the immature DP thymocyte stage of differentiation and "mature" CD8 enhancer elements drive CD8 gene expression at the mature $CD8^+$ T cell stage of differentiation. Thus, TCR signals can induce the basal transcriptional state in DP thymocytes by simply terminating activation of immature CD8 enhancer elements. It is interesting to further speculate (26) that TCR signals in developing thymocytes might block activation of all lineage-specific regulatory elements (i.e., CD8 enhancer and CD4 silencer elements) with the result that persistent TCR signaling would maintain CD4 gene expression "on" and CD8 gene expression "off" (i.e., $CD4^+8^-$), resulting in thymocyte differentiation into $CD4^+$ T cells as proposed by the kinetic signaling model.

In conclusion, this study demonstrates that the appearance of MHC-I–signaled thymocytes as $CD4^+8^{lo}$ cells results from transient cessation of CD8 gene expression. Indeed, this study demonstrates that initial cessation of CD8 gene expression does not imply commitment to the $CD4^+$ T cell lineage, as many in vivo MHC-I–signaled $CD4^+8^-$ cells are able to undergo coreceptor reversal and differentiate into $CD8^+$ T cells. Thus, these observations support the kinetic signaling model and challenge the concept that lineage commitment occurs in DP thymocytes and results in permanent silencing of the opposite coreceptor gene.

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