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Targeting CD4 Coreceptor Expression to Postselection Thymocytes Reveals That CD4/CD8 Lineage Choice Is neither Error-Prone nor Stochastic

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The mechanism by which CD4/CD8 lineage choice is coordinated with TCR specificity during positive selection remains an unresolved problem in immunology. The stochastic/selection model proposes that CD4/CD8 lineage choice in TCR-signaled CD4+CD8+ thymocytes occurs randomly and therefore is highly error-prone. This perspective is strongly supported by “coreceptor rescue” experiments in which transgenic CD4 coreceptors were ectopically expressed on thymocytes throughout their development and caused significant numbers of cells bearing MHC-II-specific TCR to differentiate into mature, CD8 lineage T cells. However, it is not known if forced coreceptor expression actually rescued positively selected thymocytes making an incorrect lineage choice or if it influenced developing thymocytes into making an incorrect lineage choice. We have now reassessed coreceptor rescue and the concept that lineage choice is highly error-prone with a novel CD4 transgene (referred to as E8I-CD4) that targets expression of transgenic CD4 coreceptors specifically to thymocytes that have already undergone positive selection and adopted a CD8 lineage fate. Unlike previous CD4 transgenes, the E8I-CD4 transgene has no effect on early thymocyte development and cannot itself influence CD4/CD8 lineage choice. We report that the E8I-CD4 transgene did in fact induce expression of functional CD4 coreceptor proteins on newly arising CD8 lineage thymocytes precisely at the point in thymic development that transgenic CD4 coreceptors would putatively rescue MHC-II-specific thymocytes that incorrectly adopted the CD8 lineage. However, the E8I-CD4 transgene did not reveal any MHC-II-selected thymocytes that adopted the CD8 lineage fate. These results demonstrate that CD4/CD8 lineage choice is neither error-prone nor stochastic. The Journal of Immunology, 2008, 181: 6975–6983.

How cell fate specification occurs in bipotential progenitor cells is an important problem in metazoan development. A mammalian example of such cell fate specification is the differentiation of bipotential CD4–CD8– double-positive (DP) thymocytes into either CD4 or CD8 lineage T cells. In the thymus, DP thymocytes are signaled by their TCR to undergo positive selection and to differentiate into either CD4+CD8− or CD4−CD8+ single-positive (SP) T cells, which constitute the major T cell arm of the adaptive immune system. Importantly, coreceptor expression in SP T cells must be matched to TCR specificity and cellular function, such that CD4+ T cells possess helper function and express TCR specific for peptides presented by MHC-II molecules, while CD8+ T cells possess cytotoxic function and express TCR specific for peptides presented by MHC-I molecules (1–3).

Understanding how TCR specificity, coreceptor phenotype, and cellular function are appropriately matched during T cell development in the thymus remains an unresolved issue. The stochastic/selection model of CD4/CD8 lineage choice postulates that CD4/CD8 lineage choice by signaled DP thymocytes is entirely random, regardless of TCR specificity, and that signaled DP thymocytes differentiate into SP cells that then require additional TCR/coreceptor “rescue” signals to differentiate into long-lived T cells (4–9). Strong support for the stochastic/selection model has come from so-called “coreceptor rescue” experiments in which forced or persistent expression of one of the coreceptor proteins resulted in the appearance of mature T cells bearing TCR with inappropriate MHC recognition specificities for their T cell lineage (7, 8, 10, 11). In the case of CD4, forced expression of transgenic CD4 coreceptor proteins resulted in the appearance of mature CD8 lineage T cells bearing mismatched MHC-II-restricted TCR (7, 10). Furthermore, genetic deletion of the CD4 silencer element (12, 13) prevented down-regulation of CD4 expression on CD8 lineage T cells and caused the generation of mature CD8 lineage T cells bearing mismatched MHC-II-restricted TCR. Such experiments were interpreted as fulfilling the central premise of the stochastic/selection model that CD8 SP thymocytes arise during MHC-II-specific positive selection but that such cells normally fail to differentiate into long-lived CD8 lineage T cells because they cannot receive rescue signals from their MHC-II-specific TCR in the absence of CD4 coreceptors (12).

Because the CD4/CD8 lineage decision is central to our understanding of T cell development in the thymus, it was important to determine whether SP thymocytes that have randomly made an
incorrect lineage choice in fact exist and whether they are in fact rescued by forced expression of the matching coreceptor protein. Previous reports of coreceptor rescue utilized experimental models in which forced expression of the rescuing coreceptor was initiated early and continuously throughout thymocyte development, so that the rescuing coreceptor might well have altered T cell selection and lineage choice in some way other than by rescuing putatively short-lived SP thymocytes. Consequently, the present study has addressed the issue of coreceptor rescue in a novel experimental model consisting of a CD4 coreceptor transgene (referred to as E8I-CD4) that targets CD4 coreceptors only to thymocytes that have already undergone positive selection and have committed to a CD8 lineage fate, precisely the point in thymocyte development that CD4 coreceptors would rescue MHC-II-specific CD8 lineage thymocytes. The present study demonstrates that the E8I-CD4 transgene does prevent the loss of CD4 coreceptor expression on CD8-committed thymocytes, but nevertheless fails to rescue or reveal significant numbers of MHC-II-specific thymocytes that had made an incorrect CD8 lineage choice. Thus, the present study directly contradicts the concept of coreceptor rescue and directly contradicts the central premise of stochastic/selection.

Materials and Methods

Mice

C57BL/6 (B6), CD40, β2m (β2-microglobulin), and OT-II TCR transgenic mice (14) were obtained from The Jackson Laboratory. 8DP4 transgenic mice bred to CD40 transgenic mice (14, 15). All mice were maintained under pathogen-free conditions in accordance with National Institutes of Health guidelines.

Generation of E8I-CD4 transgenic mice

The E8I-CD4 transgenic vector consisted of the murine 7.6-kb E8I cis-CD8 enhancer and Cdxα promoter (16) upstream of a murine CD4 cDNA. The construct was subcloned into a modified pRES-2EGFP (Invitrogen) plasmid with the IRES-EGFP sequence replaced with a poly(A) fragment. To generate a transgenic mouse, the vector transgenic plasmid was digested with Aflal and Norl, and a 13-kb fragment comprised of the E8I-CD4 transgene (see Fig. 1B) was injected into C57BL/6 (B6) pronuclei. Founder offspring were obtained and screened by Southern blot for transgene insertion and by flow cytometry of peripheral blood for CD4 expression on CD8 T cells. E8I-CD4 transgenic mice were identified by the presence of a 1.3-kb product in PCR of tail DNA using the following primers (forward: 5′-GTCCAGCTCAAGCTTCGAATT-3′, reverse: 5′-CAGTTAGACACTGCCACAGGCCC-3′) in a 32-cycle PCR reaction with an annealing temperature of 65°C.

Abs and flow cytometry

Cell suspensions were prepared from thymus and lymph nodes (LN) and stained with fluorochrome-conjugated Abs with the following specificities: CD4 (GK1.5 and RM4.5), CD69 (H1.2F3), Qa-2 (1-1-2), CD25 (7D4), TCRβ (H57-59), CD5 (53–7.3) (all from BD Pharmaning), CD8α (CT-CD8α, Caltag Laboratories), CD24 (M1/69, BD Pharmaning), and IL-7Rα (A7R34, eBioscience). OT-II transgenic TCR were identified by staining with Vα2 (B20.1, eBioscience) and Vβ5 (MH2-3D, BD Pharmaning) Abs. For FACS analysis, 1–2 × 10⁶ cells were incubated with 2.4G2 (anti-mouse FcγIII/II receptor; BD Pharmaning) and stained with fluorochrome-conjugated Abs and analyzed with a FACS Vantage SE flow cytometer (BD Biosciences). Flow cytometry data were analyzed using software developed at the National Institutes of Health and displayed in 4-decade log scale.

Intracellular calcium flux

For calcium flux analyses, 2 × 10⁶ LN cells were loaded with the calcium dye Indo-1 (Molecular Probes) at a final concentration of 1.8 μM and incubated for 30 min at 37°C. After washing, cells were incubated on ice for 40 min with 5 μg/ml biotinylated anti-TCRβ (H57-59) alone or in combination with 1 μg/ml biotinylated anti-CD4 (GK1.5) Abs, anti-CD4-FITC (RM4.5), and anti-CD8α-PE (53–6.72) (all from BD Pharmaning). Stained cells were then washed twice and prewarmed to 37°C for 2 min before being applied to the flow cytometer. Cells were signaled 30 s after being applied to the flow cytometer by cross-linking with avidin (4 μg/ml), and calcium mobilization was recorded for 4 min following cross-linking. Intracellular calcium concentrations were determined by the ratio of Indo-1 fluorescence at 405 vs 510 nm using FlowJo software (Tree Star).

Results

Experimental design and characterization of E8I-CD4 transgenic mice

The stochastic/selection model predicts that up to 50% of MHC-II-signdaled DP thymocytes randomly shut off CD4 gene expression and make an incorrect lineage decision to become CD4–8+ thymocytes with mismatching TCR and coreceptors that cannot generate TCR-mediated rescue signals (4–9) (Fig. 1A, top panel). To determine whether significant numbers of MHC-II-selected thymocytes actually select a CD8 lineage fate and die as a result, we designed a CD4 transgene (referred to as E8I-CD4) that specifically targets transgenic CD4 coreceptors to thymocytes that have undergone positive selection and have recently adopted a CD8 lineage fate. We did so by utilizing the E8I-CD8 gene-enhancer element whose activity is restricted to postselection CD8 lineage T cells (16, 17). The E8I-CD4 transgene consists of CD4 cDNA whose expression is transcriptionally controlled by the E8l enhancer and CD8α promoter (Fig. 1B), and we introduced the E8I-CD4 transgene into C57BL/6 (B6) mice to generate E8I-CD4 transgenic mice. Consequently, the E8I-CD4 transgene would rescue any MHC-II-selected T cells that adopted a CD8 lineage fate (Fig. 1A, bottom panel).

Comparison of E8I-CD4 and nontransgenic (wild-type [WT]) littermate mice revealed that overall thymus cellularity and TCRβ expression were comparable in both mice (Fig. 2A). However, analysis of CD4 and CD8 cell types revealed that CD4–8+ thymocytes that were present in WT mice (Fig. 2B, left panel, shown in blue) were largely absent in E8I-CD4 mice, which instead contained a population of CD8+ cells expressing CD4 at uniquely high levels (Fig. 2B, left panel, shown in red). Such CD4hi/CD8lo thymocytes in E8I-CD4 mice were equivalent to mature CD8 SP thymocytes in WT mice except for their expression of transgenic CD4, as both were identically CD24hi/CD8hi cells (Fig. 2B, middle panel). Because CD24hi/CD8hi cells in E8I-CD4 mice expressed transgenic CD4, we refer to them as “CD8 lineage cells” rather than referring to them as CD8 SP cells since these cells were not phenotypically CD4–8+. Indeed, CD8 lineage T cells in both the periphery (Fig. 2B, right panel) and thymus (Fig. 2B, left panel) of E8I-CD4 mice expressed high levels of transgenic CD4. However, with regard to expression of T cell markers other than CD4, CD8 lineage cells in E8I-CD4 mice were essentially identical to CD8+ T cells in WT mice (Fig. 2C).

Kinetics of E8I-CD4 transgene expression during thymocyte development

Potential rescue of mismatched MHC-II-specific CD8 lineage thymocytes requires that the E8I-CD4 transgene maintain CD4 protein expression despite down-regulation of endogenous CD4. Consequently, we assessed the ability of the E8I-CD4 transgene to maintain CD4 protein expression on developing CD8 lineage thymocytes. To relate thymocyte development to thymic selection, we defined progressive stages of CD8+ thymocyte maturation by their expression of TCRβ and CD24, as maturing thymocytes progressively increase surface expression of TCRβ and then down-regulate surface expression of CD24 (18–20) (Fig. 3, middle panels). As a result, pre-selection thymocytes are TCRβhiCD24*b3 (Fig. 3A, middle panels, gates I and II), thymocytes undergoing positive selection are TCRβhiCD24*b3 (gate III), postselection thymocytes are TCRβhighCD24high (gate IV), and maturing thymocytes are...
TCR	extsuperscript{high}CD24	extsuperscript{low} (gate V). In WT mice, it can be seen that surface CD4 expression is maintained on developing CD8\textsuperscript{+} thymocytes before and during positive selection (Fig. 3A, top right panel, gates I–III), after which surface CD4 expression progressively declines as postselection thymocytes differentiate into mature CD8 lineage T cells (Fig. 3A, top panel, gate V). In contrast, in E8\textsubscript{I}-CD4 mice, CD4 expression is maintained on developing CD8\textsuperscript{+} thymocytes throughout their selection and differentiation into mature CD8 lineage T cells,
at which point it is dramatically increased (Fig. 3A, right panel, gates I–V). Thus, CD4 surface protein expression is never lost from developing CD8 lineage thymocytes in E8I-CD4 mice.

To clearly identify the point in thymocyte development that E8I-CD4 encoded CD4 proteins are expressed, we examined CD4 expression on thymocytes from E8I-CD4 transgenic mice that lacked endogenous CD4 expression. We introduced the E8I-CD4 transgene into CD4-deficient (CD40) mice to generate E8I-CD4(CD40) mice in which all CD4 expression was from the E8I-CD4 transgene (Fig. 3B). As expected from the transcriptional activity of the E8 enhancer element, transgenic E8I-CD4 expression was essentially limited to CD8+ thymocytes (Fig. 3B, second row). Significantly, transgenic E8I-CD4 expression was first detected on TCRβhigh CD24high thymocytes in gate III undergoing positive selection and steadily increased on postselection thymocytes in gates IV and V (Fig. 3B, right panel). These data confirm that the E8I-CD4 transgene drives surface CD4 protein expression on CD8 lineage thymocytes that are down-regulating expression of endogenous CD4.

To further document that the E8I-CD4 transgene was not expressed in preselection thymocytes, we introduced the E8I-CD4 transgene into CD402m0 mice, which contain only preselection thymocytes because MHC-I- and MHC-II-specific positive selection are both impaired. Notably, the E8I-CD4 transgene was not expressed in E8I-CD4(CD402m0) thymocytes, which remained preselection cells that were CD69+ and TCRβlow (Fig. 3B, third row).

Taken together, these data demonstrate that the E8I-CD4 transgene is not expressed in preselection thymocytes, but it is first expressed in thymocytes that had undergone positive selection and adopted the CD8 lineage fate.

The E8I-CD4 transgene fails to reveal MHC-II-specific T cells adopting the CD8 lineage fate

To reveal MHC-II-selected thymocytes adopting the CD8 lineage fate, we bred the E8I-CD4 transgene into CD402m0 mice to generate E8I-CD4(CD402m0) mice. We found that thymocytes from E8I-CD4(CD402m0) mice contained few, if any, cells identified by CD24lowCD8+ expression as CD8 lineage cells, and these were no greater in frequency or absolute number than in nontransgenic CD402m0 littermates (Fig. 4A). Analysis of peripheral LN T cells similarly showed no differences in either frequency or absolute number than in nontransgenic β2m0 littermates (Fig. 4B). Thus, the E8I-CD4 transgene did not reveal or rescue MHC-II-specific thymocytes that had adopted the CD8 lineage fate.

Nevertheless, we further addressed this possibility with TCR transgenic mice. We introduced the E8I-CD4 transgene into...
mice expressing the OT-II TCR transgene that encodes Vα2+ Vβ5+ TCR that are positively selected by I-Ab thymic elements (14). In normal OT-II TCR transgenic mice, nearly all Vβ5+ T cells in the thymus and LN were CD4 lineage (i.e., CD8+ T cells), and very few Vβ5+ T cells were CD8 lineage (i.e., CD8+) T cells (Fig. 5). Notably, introduction of the E8I-CD4 transgene into OT-II TCR transgenic mice had no significant effect, as OT-II T cells in both the thymus and LN were still overwhelmingly CD4 lineage cells, with very few OT-II T cells becoming CD8 lineage T cells (Fig. 5). Thus, the E8I-CD4 transgene also failed to rescue or reveal CD8 lineage thymocytes bearing the OT-II transgenic TCR.

**E8I-CD4 transgene promotes TCR signaling in CD8 T cells**

Because the E8I-CD4 transgene failed to reveal MHC-II-specific CD8 lineage thymocytes during thymic selection of either normal...
or TCR transgenic thymocytes, we considered that CD4 molecules encoded by the E8r-CD4 transgene might be signaling defective for unknown reasons and unable to augment TCR signal transduction. However, contrary to this possibility, E8r-CD4-encoded CD4 molecules, when coengaged with surface TCR, substantially augmented TCR signaled calcium mobilization in CD8 LN T cells from E8r-CD4 transgenic mice (Fig. 6A), confirming that E8r-CD4-encoded CD4 molecules were signaling competent.

MHC-II-restricted CD8 thymocytes express the E8r-CD4 transgene

Next, we wanted to verify that the E8r-CD4 transgene would be expressed on MHC-II-specific CD8 lineage thymocytes when such mismatched cells were actually generated during thymic selection. To do so, we utilized 8DP4 experimental mice that we have previously described in detail (15). Because 8DP4 experimental mice were on a β2m−/− genetic background, their thymus only expressed MHC-II selecting elements and their thymocytes only expressed transgenic CD4 molecules. 8DP4 experimental animals...
express the E8-I-CD4 transgene that drives CD4 coreceptor expression only in preselection DP thymocytes so that CD4 expression and MHC-II-restricted TCR signaling are prematurely terminated during MHC-II-specific positive selection. As a result, MHC-II-signaled thymocytes in 8DP4 experimental mice uniformly adopt the CD8 lineage fate (15). For the present study we introduced the E8-I-CD4 transgene into 8DP4 experimental mice and analyzed CD4 expression on such MHC-II-selected CD8 lineage thymocytes (Fig. 6B). We confirmed that MHC-II-selected CD8 lineage (CD24+CD8+) thymocytes were generated both in 8DP4 mice (Fig. 6B, left panel; shown in green) and in 8DP4 mice containing the E8-I-CD4 transgene (Fig. 6B, left panel; shown in red). Most importantly, we found that the E8-I-CD4 transgene was expressed in MHC-II-selected CD8 lineage cells, as nearly all such cells were CD4+ (Fig. 6B, middle histogram). Notably, expression of CD4 coreceptors encoded by the E8-I-CD4 transgene
dramatically increased CD5 expression on mismatched MHC-II-restricted CD8 lineage thymocytes in 8DP4 mice (Fig. 6B, right histogram), indicating that E8I-CD4 encoded CD4 coreceptors augmented in vivo signaling by MHC-II-specific TCR (21). These results demonstrate that CD4 coreceptors encoded by the E8I-CD4 transgene are indeed expressed on MHC-II-restricted CD8 lineage thymocytes when such cells are generated in the thymus, and that the transgene-encoded CD4 coreceptors are functional.

While MHC-II-specific CD8 lineage T cells are generated in the thymus of 8DP4 mice, the numbers of such MHC-II-specific CD8 lineage T cells present in the periphery are reduced compared with conventional B6 CD8+ T cells (Fig. 6C), because their TCR/coreceptor mismatch renders them unable to generate homeostatic TCR signals needed for long-term survival in vivo (15, 22). Thus, we asked if the E8I-CD4 transgene, by inducing expression of matching CD4 coreceptors, would increase the number of MHC-II-specific CD8 lineage T cells present in the periphery of 8DP4 experimental mice. In fact, expression of the E8I-CD4 transgene did significantly increase the number of MHC-II-specific CD8 lineage T cells present in the periphery of 8DP4 mice and increased them to levels comparable to conventional B6 CD8+ T cells (Fig. 6C). These results indicate that the E8I-CD4 transgene is capable of rescuing MHC-II-specific CD8 lineage T cells, but that TCR/coreceptor-mediated rescue signals are not required during development in the thymus but are only required in the lymphoid periphery.

Discussion

The present study documents that thymocytes that have been positively selected by MHC-II-specific TCR rarely adopt the incorrect CD8 lineage fate. Persistence of CD4 coreceptor expression on CD8-committed thymocytes was achieved by the E8I-CD4 transgene, which specifically targeted transgenic CD4 protein expression to CD8-committed thymocytes and prevented loss of cell-surface CD4 protein expression. However, despite persistent CD4 coreceptor expression, MHC-II-selected thymocytes only rarely adopted a CD8 lineage fate. Thus, the present study directly contradicts the concept of coreceptor rescue and directly contradicts the central premise of stochastic/selection. Instead, the present study documents that CD4/CD8 lineage choice in the thymus is neither stochastic nor significantly error-prone.

The stochastic/selection model postulates that CD4/CD8 lineage choice is highly error-prone, as signaled DP thymocytes randomly terminate expression of one or the other coreceptor to become short-lived SP thymocytes that require matching TCR and coreceptors to differentiate into mature T cells. Consequently, stochastic/selection predicts that ~50% of MHC-II-signalated thymocytes maintain CD4 coreceptor expression and differentiate into mature CD4+ T cells, while the other 50% of MHC-II-signalated thymocytes terminate CD4 coreceptor expression and die (4–7, 11, 23). To directly assess this prediction, the present study utilized the E8I-CD4 transgene, which encodes CD4 coreceptor molecules that are first expressed when positively selected thymocytes have adopted the CD8 lineage fate and begun down-regulating endogenous CD4 expression, precisely the developmental point at which CD4 coreceptor rescue of MHC-II-selected CD8 lineage thymocytes would occur. In fact, the E8I-CD4 transgene functioned as designed in that it successfully induced expression of competent CD4 coreceptors on MHC-I-selected CD8 lineage thymocytes and successfully induced expression of competent CD4 coreceptors on MHC-II-selected CD8 lineage thymocytes when such cells were generated in 8DP4 experimental mice by premature termination of intrathymic MHC-II signaling. Nevertheless, the E8I-CD4 transgene did not reveal MHC-II-selected thymocytes adopting the CD8 lineage fate during normal thymic selection, indicating that MHC-II-selected thymocytes do not normally make an incorrect lineage choice.

The present study differs importantly from previous CD4 coreceptor rescue (7, 10) experiments in the timing of CD4 transgene expression during thymocyte development and in the detection of MHC-II-specific CD8 lineage T cells. Previous CD4 coreceptor rescue experiments utilized CD4 transgenes whose expression was controlled by heterologous transcriptional control elements that initiated CD4 transgene expression early and continuously throughout thymocyte development, and resulted in the appearance of MHC-II-specific CD8 lineage T cells (7, 10). We do not think that the appearance of such MHC-II-specific CD8 lineage T cells experiments was due to coreceptor rescue of short-lived MHC-II-selected thymocytes, as has been thought (7, 10). Rather, we suggest that the appearance of MHC-II-specific CD8 lineage T cells was the result of a characteristic intrinsic to CD4 coreceptor transgenes driven by heterologous transcriptional control element, namely, that their expression is down-regulated during positive selection, causing premature disruption of MHC-II signaling during positive selection of thymocytes with low-affinity, coreceptor-dependent TCR and redirecting them to differentiate into CD8 lineage T cells (S. D. Sarafova, F. Van Laethem, T. Guinter, O. S. Sharrow, L. Feigenbaum, R. Bosselut, and A. Singer, manuscript in preparation). Indeed, differentiation of MHC-II-signalated thymocytes into CD4 lineage cells requires persistent MHC-II signaling, as premature disruption of MHC-II signaling during positive selection has been shown to redirect differentiation of MHC-II-specific thymocytes into CD8 lineage T cells (15, 24).

Appearance of MHC-II-restricted CD8 lineage T cells in CD4 silencer-deficient (CD4sil−/−) mice has a similar explanation (12). Targeted deletion of the Cd4 silencer element not only removed the transcriptional control element that silences CD4 expression on CD4+ thymocytes, but it also removed a positive regulatory element (25) that augments CD4 coreceptor expression on signalated thymocytes during positive selection and differentiation into mature CD4+ T cells (12). Consequently, because CD4 coreceptor expression declined during positive selection, it is likely that MHC-II signaling was prematurely disrupted in CD4sil−/− thymocytes with low-affinity TCR, causing such MHC-II-signalated thymocytes to differentiate into CD8 lineage T cells.

We think that the CD4/CD8 lineage decision is best described by the kinetic signaling model (3, 26, 27). According to kinetic signaling, TCR-signalated DP thymocytes selectively terminate CD8 coreceptor transcription, regardless of the specificity of their TCR, to transcriptionally become CD4+CD8+ intermediate thymocytes that may phenotypically appear as CD4+CD8low cells. It is in such intermediate thymocytes that CD4/CD8 lineage choice is made, and the choice is based on whether the TCR-mediated selection signal persists or ceases in the absence of CD8 coreceptor transcription. If diminished CD8 coreceptor expression does not disrupt TCR signaling, thymocytes develop into CD4 lineage T cells; however, if diminished CD8 coreceptor expression does disrupt TCR signaling, thymocytes develop into CD4 lineage T cells (3, 26, 27). Indeed, 8DP4 experimental mice were originally constructed to test a prediction of kinetic signaling (15); that is, that loss of CD4 coreceptor expression would disrupt MHC-II signaling in intermediate thymocytes and cause them to differentiate into CD8 lineage T cells. 8DP4 experimental mice also proved invaluable in the present study because they allowed us to experimentally confirm that the E8I-CD4 coreceptor transgene was expressed on MHC-II-selected CD8 lineage thymocytes when such cells were actually generated in the thymus.
In conclusion, the present study demonstrates that CD4/CD8 lineage choice in the normal thymus is not stochastic and is not significantly error-prone, contradicting the central premise of the stochastic/selection model and contradicting the results of previous coreceptor rescue experiments. We suggest that the appearance of T cells with mismatching TCR and coreceptors in experimental circumstances does not imply an underlying stochastic mechanism to CD4/CD8 lineage choice, but rather is due to redirected differentiation of positively selected thymocytes as described by kinetic signaling (3, 15, 27).

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Disclosures
The authors have no financial conflicts of interest.

References