Commitment of Immature $CD4^+8^+$ Thymocytes to the CD4 Lineage Requires CD3 Signaling but Does Not Require Expression of Clonotypic T Cell Receptor (TCR) Chains

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Summary

As a consequence of positive selection in the thymus, immature $CD4^+8^+$ double-positive, [DP] thymocytes selectively terminate synthesis of one coreceptor molecule and, as a result, differentiate into either $CD4^+$ or $CD8^+$ T cells. The decision by individual DP thymocytes to terminate synthesis of one or the other coreceptor molecule is referred to as lineage commitment. Previously, we reported that the intrathymic signals that induced commitment to the CD4 versus CD8 T cell lineages were markedly asymmetric. Notably, CD8 commitment appeared to require lineage-specific signals, whereas CD4 commitment appeared to occur in the absence of lineage-specific signals by default. Consequently, it was unclear whether CD4 commitment, as revealed by selective termination of CD8 coreceptor synthesis, occurred in all DP thymocytes, or whether CD4 commitment occurred only in T cell receptor (TCR)–CD3-signaled DP thymocytes. Here, we report that selective termination of CD8 coreceptor synthesis does not occur in DP thymocytes spontaneously. Rather, CD4 commitment in DP thymocytes requires signals transduced by either CD3 or ζ chains, which can signal CD4 commitment even in the absence of clonotypic TCR chains.

Immature $CD4^+8^+$ (double-positive [DP])[1] cells differentiate in the thymus into mature $CD4^+8^-$ or $CD4^-8^+$ (single-positive, SP) T cells by the process of positive selection. Consequently, positive selection of DP thymocytes requires that synthesis of either CD4 or CD8 coreceptor molecules be terminated, an event referred to as lineage commitment. Several models of lineage commitment have been proposed (1–13) and have been actively debated for years. The instructional model of lineage commitment (1, 2) postulates that lineage-specific signals transduced by surface coreceptor molecules, together with TCR signals, terminate synthesis of the inappropriate coreceptor. The stochastic/selection model (3–7) postulates that TCR-signaled thymocytes randomly terminate synthesis of one or the other coreceptor molecule, and, because such cells are short-lived, they require a subsequent TCR signal to become long-lived cells that can differentiate into maturity. Both the instructional and stochastic/selection models of lineage commitment presume that identical rules govern CD4 commitment and CD8 commitment with the only difference being the identify of their target coreceptor molecule. In contrast, the asymmetric commitment model (8) postulates that commitment to the CD4 and CD8 lineages are consequences of very different signaling events. That is, CD8 commitment requires TCR and CD8-specific lineage signals, whereas CD4 commitment results from the absence of CD8-specific lineage signals.

The asymmetric commitment model was originally based on the molecular definition of lineage commitment in DP thymocytes as the selective termination of synthesis of one coreceptor molecule, and was prompted by experiments assessing coreceptor synthesis in individual DP thymocytes. By use of the coreceptor reexpression assay, we observed that commitment of DP thymocytes to the CD8 lineage (i.e., termination of CD4 synthesis) strictly required specific TCR recognition of intrathymic MHC class I determinants. In contrast, we observed that commitment of DP thymocytes to the CD4 lineage (i.e., termination of CD8

[1]*Abbreviations used in this paper:* DN, double negative; DP, double positive; SP, single positive; TSA, thymic shared antigen.

synthesis) occurred in the absence of CD8 commitment, whether or not MHC class II molecules were expressed in the thymus (8). As a result, commitment to the CD4 lineage did not require lineage-specific signals and so appeared to be a default developmental pathway. Essentially identical results were independently obtained examining in vitro cultured thymocytes (13). Other in vitro studies attempting to signal thymocyte differentiation in culture have similarly found that the requirements for $CD4^+$ T cell generation are far less stringent than those for $CD8^+$ T cell generation (14–16). In addition to signaling asymmetries, there also exist marked asymmetries in the phenotypic progression by which immature thymocytes differentiate into $CD4^+$ versus $CD8^+$ T cells in vivo (17–19).

Recently, we determined that DP thymocytes that have undergone lineage commitment have a distinct phenotype relative to the majority of DP thymocytes that have not yet undergone lineage commitment. We found that all lineage-committed DP thymocytes, including those committed to the CD4 lineage, were $CD5^{hi}CD69^{hi}$ and so had presumably experienced a TCR signaling event in the thymus, indicating that TCR–CD3 signaling was involved in thymocyte commitment to either T cell lineage (20). The present study was undertaken specifically to determine whether or not TCR–CD3 signals were required to induce lineage commitment in immature DP thymocytes. In particular, we wished to assess whether CD4 commitment, as revealed by selective termination of CD8 coreceptor synthesis in DP thymocytes, was dependent on TCR–CD3 signaling or whether it occurred spontaneously in immature DP thymocytes.

Materials and Methods

Animals. All mice were housed and bred in a specific pathogen-free facility and used at 4–12 wk of age. TCRα° mice (21) were obtained from The Jackson Laboratory (Bar Harbor, ME). TCRζ° mice (22) were mated with RAG2° mice (23) to generate double-deficient RAG2°TCRζ° mice. Mice transgenic for a chimeric protein consisting of the extracellular and transmembrane domains of human CD25 and the cytosolic domain of murine TCRζ (TTζ, line no. 35) (24) were also bred onto the RAG2° background to make RAG$^\circ$–TTζ mice.

Generation of DP Thymocytes in RAG2° Mice. RAG2° and RAG2$^\circ\zeta^\circ$ mice were injected intraperitoneally with 250 μg of affinity-purified anti-CD3ϵ mAb (145-2C11) (25) or with the dose indicated. RAG$^\circ$–TTζ mice were injected with 250 μg anti-Tac mAb (1HT4-4H3). 8 d after injection, thymocytes were obtained and analyzed by flow cytometry. Where indicated, RAG2° mice were injected with 250 μg of affinity-purified anti-CD3ϵ mAb on both day 0 and day 8 and then subjected to the coreceptor reexpression assay on day 12. Where indicated, RAG2° mice were radiated with 400 cGy and analyzed 3 wk later (26, 27).

Cell Sorting and Coreceptor Reexpression Assay. Performance of the coreceptor reexpression assay on electronically sorted thymocyte populations has been described previously (8). In brief, single cell suspensions of thymocytes were stained with PE-conjugated anti-CD4 mAb (GK1.5; Becton Dickinson, San Jose, CA) and FITC-conjugated anti-CD8 mAb (53-6-72, Becton Dickinson). Stained thymocytes were electronically sorted by a FACstar® Plus according to the gates indicated in each figure. Sorted cells were washed extensively with PBS and treated with 0.04% pronase (Calbiochem Novabiochem, San Diego, CA) and 100 μg/ml DNase I (Boehringer Mannheim, Indianapolis, IN) at 37°C for 15 min, pelleted, and pronase treated for another 10 min at 37°C. Cells were placed in culture for 12–16 h of culture at either 4 or 37°C, after which harvested cells were restained with anti-CD4–PE and anti-CD8–FITC. For three-color analysis cells were also stained with anti-CD5 (53-7-3; PharMingen) followed by Cy-5 avidin (Caltag, San Francisco, CA). Dead cells were excluded by electronic gating on both forward light scatter and propidium iodide intensity. Flow cytometry using three- or four-decade logarithmic amplification as indicated was performed on a FACStar® Plus and data were analyzed using software designed by the Division of Computer Research and Technology at the National Institutes of Health.

Results

To assess the possibility that DP thymocytes spontaneously terminated CD8 coreceptor synthesis even in the absence of TCR–CD3 signals, we examined DP thymocytes from TCRα° mice by the coreceptor reexpression assay. TCRα° thymocytes cannot express conventional $\alpha\beta$ TCR complexes and, consequently, cannot differentiate beyond the DP stage of development (21). To enrich for DP thymocytes that might have committed to the CD4 or CD8 T cell lineages, we electronically sorted for $CD4^+8^{lo}$ and $CD4^{lo}8^+$ transitional cell populations and utilized the coreceptor reexpression assay to determine the coreceptor molecules they were actively synthesizing (Fig. 1, *A* and *B*). In the coreceptor reexpression assay, preexisting surface CD4 and CD8 coreceptor molecules are removed from the sorted cells by treatment with low doses of pronase, and the stripped cells then placed into single cell suspension cultures for 14 h. Metabolic activity of cultured cells is inhibited at 4°C, so that cell surface coreceptor reexpression does not occur (Fig. 1 *A* and *B*, *middle columns*). However, cells cultured at 37°C do reexpress the CD4 and/or CD8 coreceptor molecules that they are actively synthesizing. Indeed, we have previously demonstrated that coreceptor reexpression in this assay requires active coreceptor transcription and protein synthesis (8). Interestingly, virtually all $CD4^+8^{lo}$ sorted cells from TCRα° DP thymocytes reexpressed both CD4 and CD8 coreceptors and so reappeared as DP cells (Fig. 1 *A*, *top*). Identical results were obtained with $CD4^{lo}8^+$ sorted cells from TCRα° mice (Fig. 1 *B*, *top*). That is, none of the DP thymocytes present in TCRα° mice had selectively terminated either CD4 or CD8 coreceptor synthesis, indicating that none had undergone lineage commitment. Thus, these results indicated that lineage commitment did not occur spontaneously in immature DP thymocytes but might be dependent upon signals transduced by surface TCR–CD3 complexes.

To assess directly the role of TCR–CD3 signals in inducing lineage commitment in DP thymocytes, we assessed DP thymocytes from experimentally induced RAG2° mice. RAG2° mice fail to express any clonotypic TCR chain because they are unable to recombine any TCR gene locus.

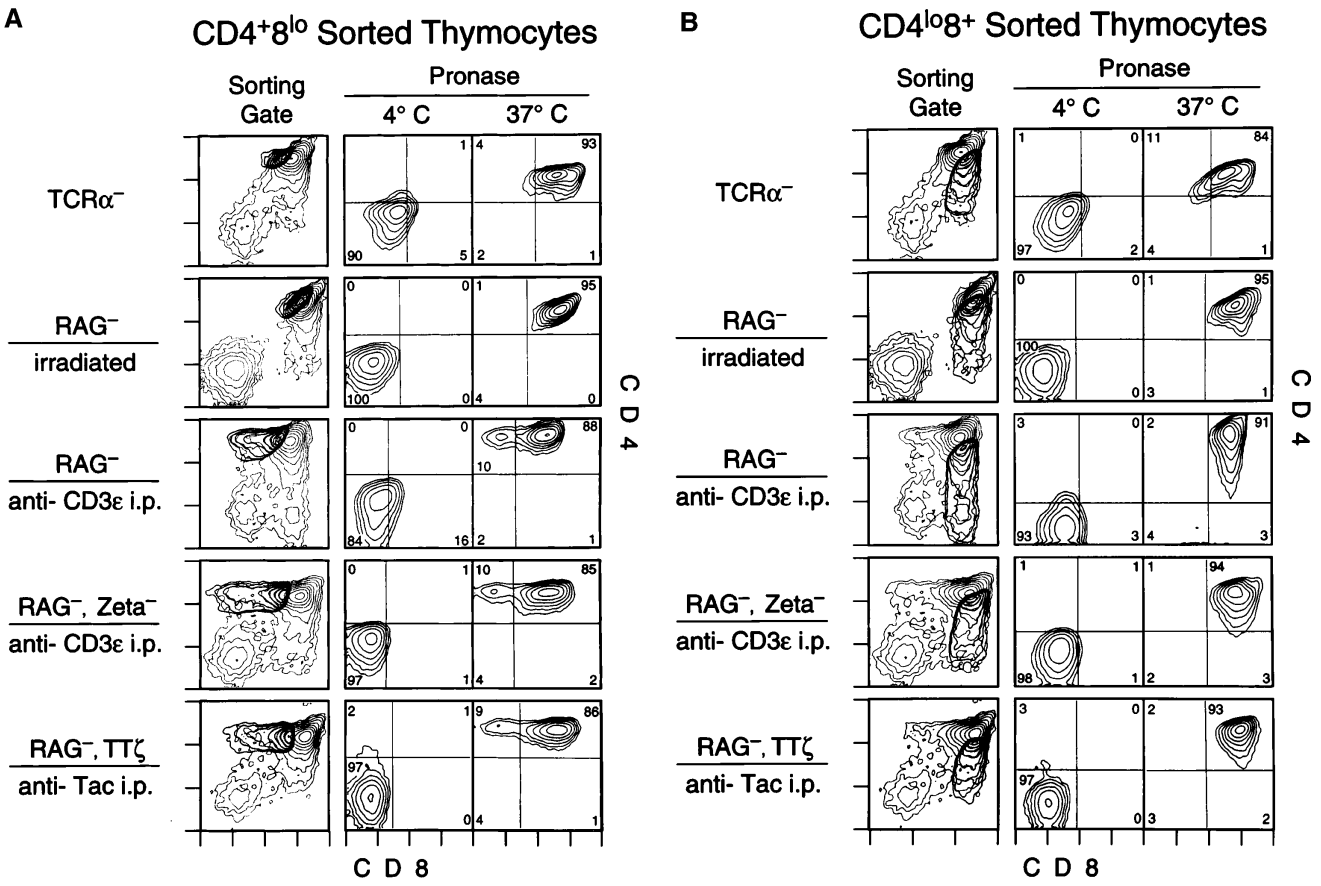

Figure 1. Lineage commitment in sorted $CD4^{+}8^{lo}$ and $CD4^{lo}8^{+}$ thymocytes from experimental mice. Purified populations of $CD4^{+}8^{lo}$ thymocytes (*A*) and $CD4^{lo}8^{+}$ thymocytes (*B*) were obtained by electronic cell sorting according to the indicated sorting gates superimposed on the starting thymocyte populations (*left columns*). Sorted thymocyte populations were stripped of surface coreceptor molecules by treatment with low doses of pronase, after which they were placed in suspension cultures at 4°C (*middle panels*) or 37°C (*right panels*) for 12–16 h and restained for CD4 and CD8 surface expression. The coreceptor reexpression assay detects the coreceptor molecules that individual thymocytes synthesized during the 37°C culture, and is dependent upon new transcription and new protein synthesis (8). Sorted thymocytes that reexpress both CD4 and CD8 coreceptor proteins are lineage-uncommitted cells; those reexpressing only CD4 are CD4 committed; and those reexpressing only CD8 are CD8 committed. Cells cultured at 4°C do not reexpress surface coreceptor molecules so that their CD4–CD8 histograms reflect whatever coreceptor molecules that potentially remain after pronase treatment (*middle columns*). As we have previously described (8), the anti-CD4 mAb used to prepare thymocytes for cell sorting minimally interferes with stripping of surface CD4 molecules by pronase, resulting in a small number of residual CD4 molecules remaining on the cell surface. Consequently, to highlight changes in coreceptor reexpression during 37°C cultures, histogram boxes were drawn based on the 4°C profiles of each sorted and pronase-stripped cell population. The frequency of cells in each box is indicated. The number of the thymocytes obtained in these experimental mice were the following: TCRα° (9×10^7 cells), γ-irradiated RAG2° (4×10^7 cells), anti-CD3ϵ mAb–injected RAG2° (1.1×10^8 cells), anti-CD3ϵ mAb–injected RAG°TCRζ° (8×10^7 cells), and anti-Tac mAb–injected RAG°–TTζ (4×10^7 cells).

As a result, RAG2° thymocytes are arrested at the $CD4^{-}8^{-}$ (double-negative, DN) stage of development (23). However, RAG° thymocytes can be induced to differentiate further into DP cells by either sublethal γ-irradiation (26, 27) or by injection of anti-CD3ϵ mAb (28, 29) (Fig. 1, *A* and *B*, *left column*). Induced RAG° DP thymocytes did not further differentiate into phenotypically mature T cells as thymocytes from stimulated RAG° mice that appeared $CD4^{+}8^{-}$ or $CD4^{-}8^{+}$ (Fig. 1, *A* and *B*, *left columns*) were predominantly precursor cells that spontaneously became $CD4^{+}8^{+}$ in overnight culture (data not shown; reference 30). Even though sublethal γ-irradiation and anti-CD3ϵ injection both induced generation of RAG° DP thymocytes (Fig. 1), the two modes of stimulation did not have identical effects. That is, sublethal γ-irradiation did not detectably stimulate CD3 signal transduction as revealed by the absence of CD5 upregulation, whereas injection of anti-CD3ϵ mAb did stimulate CD3 signal transduction and CD5 upregulation (Fig. 2). Assessment of $CD4^{+}8^{lo}$ and $CD4^{lo}8^{+}$ sorted cells from γ-irradiated RAG2° mice by the coreceptor reexpression assay revealed that none had undergone lineage commitment (Fig. 1, *A* and *B*). In contrast, assessment of $CD4^{+}8^{lo}$ sorted cells from anti-CD3ϵ–induced RAG2° mice revealed the presence of CD4-committed DP thymocytes that had selectively terminated CD8 coreceptor synthesis, as well as the presence of uncommitted DP thymocytes (Fig. 1 *A*). The CD4-committed thymocytes that were present in anti-CD3ϵ–induced RAG2°

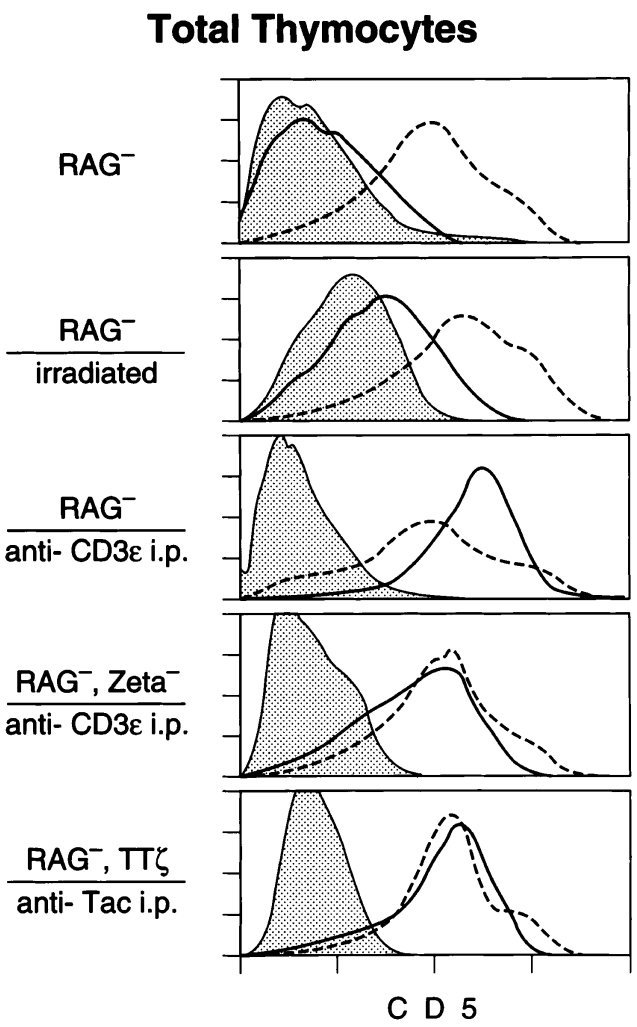

Figure 2. CD5 surface expression on thymocytes from experimental mice. Thymocytes from the indicated experimental mice were stained with anti-CD5 mAb (*solid line*) or an irrelevant antibody (*shaded curve*). CD5 expression on normal B6 thymocytes stained at the same time as a positive control is also shown for comparison (*dotted line*).

mice expressed the phenotype of newly committed thymocytes in that they were HSA^{hi}, thymic shared antigen (TSA)-1^{hi} (data not shown). In contrast, anti-CD3ϵ–induced RAG2° thymocytes had no CD8-committed cells among either $CD4^+8^{lo}$ or $CD4^{lo}8^+$ sorted cell populations (Fig. 1, *A* and *B*). Thus, these results (*a*) confirm that DP thymocytes do not undergo lineage commitment in the absence of TCR–CD3 signals, and (*b*) demonstrate that CD3 signals stimulated by anti-CD3ϵ mAbs are sufficient to induce DP thymocytes to selectively terminate CD8 coreceptor synthesis and commit to the CD4 lineage, even in the absence of clonotypic TCR chains.

To determine whether signals transduced by TCRζ chains are indispensable for induction of CD4 commitment, we generated double knockout RAG2°TCRζ° mice. In vivo injection of anti-CD3ϵ mAbs into RAG°TCRζ° double knockout mice induced the generation of DP thymocytes, as has been described (31), and signaled these cells to upregulate CD5 expression (Fig. 2). Interestingly, we found that $CD4^+8^{lo}$ sorted thymocytes from these anti-CD3ϵ–injected mice did contain CD4-committed cells that had selectively terminated CD8 coreceptor synthesis (see Fig. 1 *A*). In contrast, no CD8-committed cells were detected in either $CD4^+8^{lo}$ or $CD4^{lo}8^+$ sorted cell populations (Fig. 1, *A* and *B*). These results demonstrate that CD3-transduced signals can induce CD4 commitment in DP thymocytes in the absence of clonotypic TCR chains and in the absence of TCRζ chains.

To determine whether signals transduced by TCRζ chains were able to induce CD4 commitment, we assessed lineage commitment in DP thymocytes from RAG2° mice that expressed a chimeric transgenic protein consisting of the external and transmembrane domains of human CD25 and the cytosolic domains of TCRζ (24). The extracellular domain of this transgenic protein is recognized by anti-Tac mAb. Injection of anti-Tac mAb into RAG°–TTζ mice induced the generation of DP thymocytes (Fig. 1 *A*), as previously reported (24), and signaled them to upregulate CD5 expression (Fig. 2). We then assessed DP thymocytes from anti-Tac–induced RAG°–TTζ mice for the presence of lineage committed cells. We found that $CD4^+8^{lo}$ sorted thymocytes from these mice did contain CD4-committed cells, but did not contain any CD8-committed cells in either $CD4^+8^{lo}$ or $CD4^{lo}8^+$ sorted cell populations (see Fig. 1, *A* and *B*). These results demonstrate that signals transduced by the cytosolic portion of TCRζ chains are sufficient to induce DP thymocytes to selectively terminate CD8 coreceptor synthesis and to undergo CD4 commitment.

Next, we wished to evaluate the relationship between CD5 upregulation and CD4 commitment in anti-CD3ϵ–signaled RAG2° thymocytes. In vivo injection of a single dose of either 10 or 250 μg of anti-CD3ϵ mAb–induced substantial numbers of DP thymocytes in RAG° mice when assayed 8 d later (Fig. 3). While both injection doses induced differentiaton to the DP stage, we reasoned that only the high dose might persist long enough in vivo to stimulate a subsequent CD3 signal after DP thymocytes appeared. Indeed, only DP thymocytes from high dose injected animals had upregulated CD5 expression, and only DP thymocytes from high dose injected animals contained CD4-committed thymocytes (Fig. 3). DP thymocytes from low dose injected animals were $CD5^{lo}$ and remained uncommitted (Fig. 3). We conclude that CD4 commitment requires CD3 signals in DP thymocytes that are of sufficient intensity to upregulate surface CD5 expression.

Finally, having observed that a single injection of antibody was sufficient to stimulate CD3 or TCRζ chains to transduce signals that upregulated CD5 surface expression and induced CD4 commitment but not CD8 commitment, we assessed whether CD8-committed cells might appear in RAG2° thymi upon antibody restimulation. In Fig. 4, RAG2° mice were injected with anti-CD3ϵ mAb on both days 0 and 8, and then assessed 4 d later on day 12. Thymocytes were sorted into $CD4^+8^{lo}$ and $CD4^{lo}8^+$ pop-

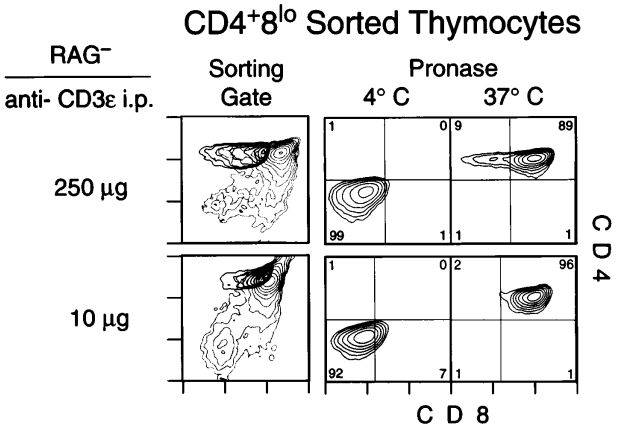

Figure 3. Correlation between induction of CD4 commitment and surface CD5 upregulation. RAG° mice received one intraperitoneal injection of either 250 μg or 10 μg of affinity-purified anti-CD3ε mAb. 8 d later, thymocytes were sorted according to the indicated gates and assessed for lineage commitment by the coreceptor reexpression assay (*left panels*). Unsorted thymocytes were placed in culture for 12 h at 4°C, at which temperature thymocyte phenotype is stable, and then assessed for surface CD5 expression (*right panels*). Solid line indicates CD5 expression on experimental thymocytes. Shaded curve represents negative control staining of experimental thymocytes with an irrelevant mAb. The dotted line represents CD5 expression on normal B6 thymocytes stained at the same time as a positive control and is shown for comparison. The number of thymocytes obtained on day 8 were 1–1.5 × 10^8 cells and 3–4 × 10^7 cells with the injection of 250 μg and 10 μg, respectively.

ulations and then assessed for coreceptor synthesis by the coreceptor reexpression assay. We found that $CD4^+8^{lo}$ thymocytes contained CD4-committed cells that had selectively terminated CD8 coreceptor synthesis (Fig. 4, *middle row*), but neither sorted population contained CD8-committed cells (Fig. 4, *middle and bottom rows*). Thus, CD8-committed cells did not appear in RAG2° thymi despite a second antibody injection and despite assessment on day 12 after the initial injection of antibody (Fig. 4). Indeed, we also failed to detect CD8-committed thymocytes on days 28 and 35 after antibody injection (data not shown).

Discussion

The present study demonstrates that immature DP thymocytes do not spontaneously terminate synthesis of either CD4 or CD8 coreceptor molecules. Rather, selective termination of coreceptor synthesis by immature DP thymocytes requires signals transduced by either CD3 or TCRζ chains, and can occur in signaled DP thymocytes that lack clonotypic TCR chains. Interestingly, CD3-signaled DP thymocytes upregulated CD5 expression and selectively terminated CD8 coreceptor synthesis, but did not selectively terminate CD4 coreceptor synthesis. Thus, CD4 commitment is induced in DP thymocytes by CD3 signals that are of sufficient intensity to upregulate CD5 expression.

The results of the present study are not readily compatible with either instructional (1, 2) or stochastic/selection (3–7) models of lineage commitment. That is, the instructional model cannot explain the presence of any lineage-committed RAG2° thymocytes in response to lineage-neutral CD3 and TCRζ signals, whereas the stochastic/selection model cannot explain why CD3 and TCRζ signals only induced RAG2° thymocytes to become CD4 committed without inducing an equal number to become CD8 committed. In contrast with these two models of lineage commitment, the present results are concordant with the asymmetric commitment model of lineage commitment (8, 13). That is, our results are consistent with the concept that CD4 commitment, unlike CD8 commitment, can occur in the absence of lineage-specific signals. Importantly, the present results extend the asymmetric commitment model by demonstrating that CD4 commitment does not occur spontaneously in unsignaled DP thymocytes, but rather is induced

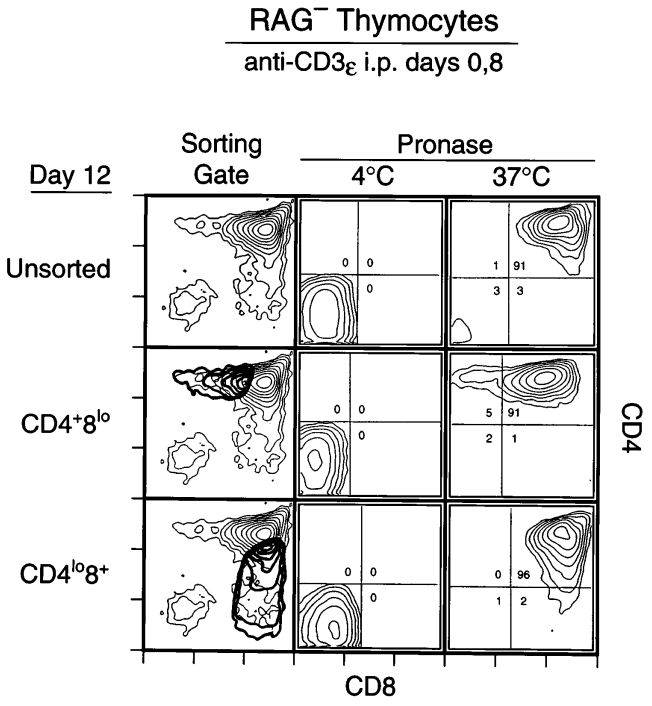

Figure 4. CD8-committed thymocytes do not appear in RAG2° thymi even after two injections of anti-CD3 mAb. RAG2° mice were injected with 250 μg of affinity-purified anti-CD3ε mAb on both day 0 and 8. On day 12, thymocytes were harvested, sorted into $CD4^+8^{lo}$ and $CD4^{lo}8^+$ populations, and assessed by the coreceptor reexpression assay for appearance of CD4-committed and CD8-committed cells. Numbers in each box represent the frequency of thymocytes falling in that box.

by lineage-neutral signals transduced by CD3 and/or TCRζ chains.

Importantly, we found that CD3 signaling did not induce all DP thymocytes in the present study to become CD4 committed, as only a small minority of CD3-signaled RAG2° thymocytes terminated CD8 coreceptor synthesis, even though all DP thymocytes had upregulated CD5 surface expression. This observation is consistent with our recent finding that only a small fraction of $CD5^{hi}$ DP thymocytes in normal mice have undergone lineage commitment, with most $CD5^{hi}$ DP thymocytes remaining lineage uncommitted (20). Our current perspective is that CD3 signaling drives $CD5^{lo}$ DP thymocytes to become $CD5^{hi}$, at which point they developmentally await the induction of lineage-specific signals. If lineage-specific signals are generated, perhaps by Notch proteins (12), $CD5^{hi}$ DP thymocytes terminate CD4 coreceptor synthesis and become CD8 committed. But if lineage-specific signals are not generated, $CD5^{hi}$ DP thymocytes terminate CD8 coreceptor synthesis and become CD4 committed. We do not know how long $CD5^{hi}$ DP thymocytes await the appearance of lineage-specific signals before terminating CD8 coreceptor synthesis, and we do not know whether there are intrathymic signals that regulate the timing of this event.

Finally, the present results are remarkable in their demonstration that CD3-signaled DP thymocytes could undergo CD4 commitment even in the absence of clonotypic TCR chains. Of course, DP thymocytes express surface molecules in addition to clonotypic TCR chains that can stimulate CD3 signaling, such as CD2, CD5, Thy-1, and Ly6 (32–34). Consequently, it is conceivable that engagement of such nonclonotypic molecules by intrathymic ligands can inefficiently mimic clonotypic TCR chains in their ability to stimulate CD3 signals that induce $CD5^{lo}$ DP thymocytes to become $CD5^{hi}$, and so eventually to become CD4 committed. The absence of $CD5^{hi}$ DP thymocytes and CD4-committed cells in TCRα° mice does not rule out intrathymic signaling by nonclonotypic molecules because surface CD3 expression is probably too low on TCRα° thymocytes to transduce such signals. Indeed, stimulation of CD3 signals by nonclonotypic receptors may provide one explanation for the appearance of small numbers of CD4-committed DP thymocytes in MHC-deficient thymi (8).

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